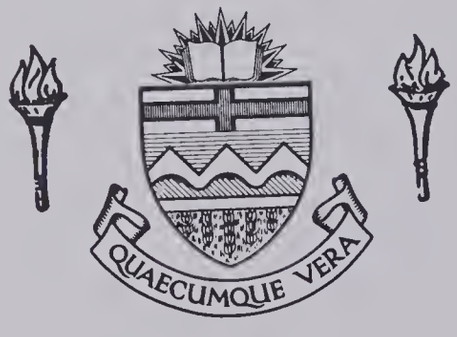

THE UNIVERSITY OF ALBERTA

CALCIUM UPTAKE AND ATPase ACTIVITY OF SARCOPLASMIC RETICULUM

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "CALCIUM UPTAKE AND ATPase ACTIVITY OF SARCOPLASMIC RETICULUM" submitted by DAVID FRANKEL in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The objectives of this study were to examine the nature of the relationship between uptake of Ca^{2+} ions and "Ca^{2+} activated" ATPase of sarcoplasmic reticulum. Initially it was shown that the biochemical properties of sarcoplasmic reticulum preparations used in this study were similar to those described by other investigators. It was demonstrated that in the presence of ATP or other nucleoside triphosphates and Mg^{2+}, calcium ions were taken up, and this uptake was greatly enhanced when oxalate was present in the reaction system. These initial experiments also demonstrated that sarcoplasmic reticulum sustained a low rate of ATP hydrolysis in the absence of Ca^{2+}, but when Ca^{2+} was added to the reaction system, the rate of ATP hydrolysis was increased. The extra ATP splitting which was observed upon Ca^{2+} addition is termed the "Ca^{2+} activated" ATPase activity.

After these initial experiments, the nature of the relationship between Ca^{2+} uptake and "Ca^{2+} activated" ATPase was investigated. The approach which was used, was to examine the degree of parallelism of these two processes under different conditions. It was observed that Ca^{2+} uptake and "Ca^{2+} activated" ATPase were inhibited in a parallel manner, when sarcoplasmic reticulum was preincubated with the sulfhydryl reagents N-Ethylmaleimide or salyrgan. These findings suggest that these two processes may be closely related, but the nature of this relationship cannot be determined. Although preincubation of sarcoplasmic

reticulum with quinidine and procaine also resulted in an inhibition of both Ca^{2+} uptake and "Ca^{2+} activated" ATPase, Ca^{2+} uptake was reduced to a greater extent than "Ca^{2+} activated" ATPase. The data for experiments with quinidine and procaine suggests that there may be a partial uncoupling of Ca^{2+} uptake from "Ca^{2+} activated" ATPase, but additional detailed studies are necessary to determine whether this observed dissociation is due to an action of these agents which increases the permeability of the sarcoplasmic reticulum vesicles to calcium.

When the effects of temperature were studied it was shown that the Arrhenius plot for Ca^{2+} uptake was linear over the temperature range of 0° - 35°C. In contrast the Arrhenius plot for "Ca^{2+} activated" ATPase is clearly non-linear over this same temperature range, and appears to be composed of two lines which intersect at about 10°C. The different temperature dependencies of Ca^{2+} uptake and "Ca^{2+} activated" ATPase again suggest that these two processes are dissociable, however alternative explanations can be put forward which are discussed in text.

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CHAPTER I

GENERAL INTRODUCTION

A) Role of Sarcoplasmic Reticulum in Skeletal Muscle Relaxation and Contraction

Kielley and Myerhoff (1948, 1949) first isolated and partially characterized an ATPase fraction from skeletal muscle (Kielley - Myerhoff's granular ATPase) which was distinct from myosin or actomyosin ATPase. The activity of this preparation accounted for more than 10% of the total ATPase of skeletal muscle. Although Kielley and Myerhoff did not recognize the significance of their findings, the fraction they isolated proved to be essentially the "relaxing factor" or fragmented sarcoplasmic reticulum fraction of muscle.

Marsh (1951, 1952) used a model system of myofibrils suspended in a muscle homogenate (muscle brei) to study the effects of ATP on fibre volume. His findings indicated the presence of a labile factor ('Marsh' factor) which prevented syneresis or contraction of myofibrils in the presence of ATP, and also markedly decreased ATP breakdown by myofibrillar ATPase. These experimental results suggested the presence of a factor in the muscle extract which was important in initiating muscle relaxation.

In order to further clarify the functional importance of the 'Marsh' factor, it was necessary to isolate a stable form of the factor, and to test its effects on appropriate muscle systems. Bendall (1952, 1953) observed that addition of his purified stable factor to glycerol treated fibres

markedly reduced the ATP splitting and shortening normally observed when ATP, $MgCl_2$ and KCl are added to this system. These results qualitatively verified Marsh's original observations. Quantitative estimations, however, of the factor's inhibiting effects on extracted fibres, must be interpreted with great caution because the thickness of the fibre does not permit even distribution of ATP throughout the fibre. Since the inhibiting effect of the "relaxing" factor depends on the ATP concentration, a true measure of its inhibition of the whole fibre system cannot be attained. Another disadvantage is that extracted fibres are usually contaminated to a certain degree with "relaxing" factor.

Another approach used to study the effects of the 'Marsh' factor has involved the use of myofibrils by Hasselbach and Weber (1953) and Portzehl (1957). Their results further substantiated those previously mentioned, since both the ATPase activity and shortening of the myofibrils were considerably reduced by addition of the factor. An advantage of using these myofibrils is that ATP can equilibrate throughout the system. It should be noted, however; that even here the myofibrils are usually somewhat contaminated with "relaxing" factor.

These early findings indicated the existence of a factor in skeletal muscle, which could at least partially account for relaxation. An important question to be answered, however, was how did the relaxing factor produce

its effects? The two alternative hypotheses advanced by investigators were that either the relaxing factor interacted directly with the contractile elements, or it removed a substance which was necessary to allow for contraction.

Since the "relaxing" factor was found to be particulate in nature (Portzehl 1957), the possibility that it interacted directly with the actomyosin system seemed unlikely. Nevertheless, several investigators isolated a non-dialyzable soluble substance from relaxing factor, which they claimed could interact directly with the contractile apparatus to produce relaxation (Parker and Gergely 1960, and Briggs and Fuchs 1960). Several problems associated with this hypothesis were that the substance could not be purified and it did not function at low ATP concentrations. In addition its relaxing effects were inconsistent, and they were less pronounced than those produced by the "relaxing" factor. Finally the most important observation which raised doubts about whether this substance had any functional significance in skeletal muscle, was the finding that relaxation could be entirely accounted for by another process (Seidel and Gergely 1964).

In order to ascertain whether relaxation can be accounted for by removal of a substance from the contractile elements, it is necessary to examine some of the effects of calcium ion on muscle functions. It has been known for a long time that Ca^{2+} plays an important role in muscle

contraction. Heilbrunn and Wiercinski (1947) injected various ions into isolated frog skeletal muscle fibres, and observed that Ca^{2+} was the only physiologically significant ion able to cause contraction. Furthermore the actual amount of Ca^{2+} required to elicit this shortening of muscle is very low. Portzehl et al (1964) used a Ca - EGTA buffer system, and showed that the threshold amount of Ca^{2+} required for shortening of a single crab skeletal muscle fibre was between .3 - 1.5 μ Moles.

In their studies of isolated actomyosin or myofibril systems, Weber and Herz (1963) found that by carefully adjusting the level of Ca^{2+} in the surrounding medium, they could control the amount of Ca^{2+} bound to the contractile elements. They determined that below a Ca^{2+} level of .2 μ Moles, there was minimal binding and relaxation, whereas at a Ca^{2+} level of 5 - 10 μ Moles, 1 - 2 moles of Ca were bound per mole of myosin, and maximal contractile-like effects and ATPase activities were observed.

Data from the different experiments of Heilbrunn, Portzehl and Weber, seemed to suggest that the level of Ca^{2+} in the vicinity of the contractile proteins is a critical factor in determining whether the muscle is in a contracted or a relaxed state. Therefore a logical experimental approach used by investigators, has been to determine whether the "relaxing" factor can sufficiently lower the level of Ca^{2+} to produce relaxation.

Perhaps the first clue which indicated that there was a relationship between the relaxing factor and Ca^{2+}, was provided by Marsh's experiments (1952). Marsh observed that the addition of Ca^{2+} to his muscle brei system immediately reversed the inhibitory effect of his postulated labile ('Marsh') factor.

The use of chelating agents which produce some of the effects of the relaxing factor, has provided valuable information concerning how the relaxing factor functions. Bozler (1954) and Watanabe (1955) observed that ethylenediamine tetra-acetic acid (EDTA) caused relaxation of glycerol-extracted fibres in the presence of Mg^{++} and ATP. Furthermore the addition of Ca^{2+} to this system reversed the relaxation produced by EDTA. On the basis of these observations Bozler and Watanabe postulated that both EDTA and the "relaxing" factor combined directly with the actomyosin system to cause relaxation, but that the addition of Ca^{2+} somehow inactivated these relaxing agents and reversed their effects.

However subsequent investigations with chelating agents provided strong evidence in favour of a different mechanism of action from that proposed by Bozler and Watanabe. Ebashi (1960) examined the relaxing effects of various chelating agents on glycerol extracted fibres. He found that the ability of these agents to cause relaxation correlated very well with their Ca^{2+} binding capacities. In a later study using ^{14}C

labelled EDTA, Ebashi (1961) showed that EDTA does not bind to actomyosin. Information derived from these studies thus indicated that the relaxing effects produced by various chelating agents is not due to binding of these agents to actomyosin, but is caused by their ability to remove Ca^{2+} from actomyosin. Also it is apparent that those agents which have the greatest binding capacities for Ca^{2+} will produce the most pronounced relaxation effects.

A number of investigators have now shown that the "relaxing" factor can bind or accumulate Ca^{2+}. The findings of Ebashi and Lipmann (1962) and Hasselbach and Makinose (1962) indicated that the "relaxing" factor could concentrate Ca^{2+} from the surrounding medium, and that this was an ATP-linked process. Furthermore Ebashi (1961) showed that relaxation of isolated actomyosin systems was due to removal of Ca^{2+} from the contractile elements, and these processes were intimately related to the binding capacities of the "relaxing" factor or chelating agents used. Weber et al (1963) had determined that Ca^{2+} bound to actomyosin was exchangeable and in equilibrium with Ca^{2+} of the surrounding medium. In addition they showed that the "relaxing" factor was able to cause dissociation of Ca^{2+} from actomyosin and effect relaxation by lowering the concentration of ionized calcium of the surrounding medium.

The studies which have so far been described, have shown that the isolated "relaxing" factor can initiate the 'in

vitro' relaxation of a variety of muscle preparations, by means of its calcium accumulating ability. Nevertheless, an important question to be considered is, what is the physiological relevance of this "relaxing" factor within the intact muscle?

The use of electron microscopy proved to be extremely valuable in the identification of the morphological components of the muscle cell from which the "relaxing" factor originated. In their electron microscopic examination of the "relaxing" factor, Ebashi and Lipmann (1962) observed that the predominant structural features consisted of vesicles and tubules, and that there was no nuclei, myofilaments or mitochondria present. The shape and size of these structures were very similar to the vesicles and tubules of the sarcoplasmic reticulum of an intact muscle fibre (Porter and Palade 1957). On the basis of these observations Ebashi and Lipmann concluded that the "relaxing" factor consisted of 'healed' or 'closed' fragments of sarcoplasmic reticulum. Muscatello et al (1962) showed that the sarcotubular fraction was the only extractable fraction from muscle which was capable of relaxing isolated myofibrils.

The findings of Ebashi and Muscatello indicated that the relaxing factor was fragmented sarcoplasmic reticulum, and that the sarcoplasmic reticulum was the physiological muscle component responsible for relaxation 'in vivo' as well as 'in vitro'.

In order to further verify this postulated physiological

role of the sarcoplasmic reticulum, a number of investigators attempted to demonstrate the 'in vivo' functioning of the sarcoplasmic reticulum. In an electron microscopic investigation of glycerinated frog muscle fibres, Hasselbach (1964a) showed that in relaxed fibres, calcium in the form of calcium oxalate was precipitated in the lateral (or terminal) sacs of the sarcoplasmic reticulum. Constantin et al (1965) used single frog muscle fibres which had their sarcolemma removed (Natori preparation). The advantages of this preparation are that it is a living fibre, in which the internal structures remain intact, and thus it is a very suitable preparation for application of various solutions to internal structures, without the interference of the surface membrane. Constantin et al observed that there was a local contraction followed by relaxation, after Ca^{2+} application to the fibre. In addition electron micrographs of fibres perfused first with Ca^{2+} and then with oxalate, revealed that the accumulation of calcium oxalate was localized in the terminal sacs of the sarcoplasmic reticulum. The results of Constantin's experiments were especially significant, since they demonstrated that the calcium accumulating properties of isolated and fragmented sarcoplasmic reticulum, operated in the fully organized sarcoplasmic reticulum of the essentially intact muscle fibre.

Much of the subsequent research on sarcoplasmic reticulum has therefore been concerned with defining its

functional role in relaxation, and it is now believed that the sarcoplasmic reticulum is vitally important in the contraction of skeletal muscle.

Hill (1949) determined that most of the cross section of a striated 50-100 μ diameter muscle fibre is activated during a single twitch. He calculated that diffusion of an "activator" substance from the fibre surface throughout the fibre interior, would be too slow to account for the short latency time between excitation of the surface membrane and the full development of the active state. In order to account for the rapidity of the mechanical response after excitation of the surface membrane, a number of investigators attempted to locate a specialized internal conduction system within the muscle. Huxley and Taylor (1958) by means of a micropipette, applied a current to small areas of the surface membrane of lizard and frog muscle fibres. Reversible contractions which spread transversely toward the fibre centre, occurred only at the level of the triad region in both types of muscle fibres. The triad had been identified by Porter and Palade (1957) as an internal membrane system consisting of two distended vesicular portions of sarcoplasmic reticulum (terminal sacs) and a tubular element which separated the sacs. The triads had a structural pattern which repeated with respect to each sarcomere of the muscle fibre.

The central element of the triad, the so-called "T" system, was found to be continuous with the sarcolemma

(Huxley 1964, Smith 1966), and it was therefore postulated that the "T" system rapidly conducted the excitation throughout the fibre. Selective disruption of the "T" system (Howell 1969), prevented the contraction which was normally observed after depolarization of the muscle surface membrane. This experiment further confirmed the role of the "T" system in coupling the electrical excitation and the mechanical response of skeletal muscle.

The proximity of the "T" system to the lateral sacs of the sarcoplasmic reticulum, suggested that the triad area was the site where coupling between excitation and contraction occurred. The postulated mechanism for this coupling was that transmission of the depolarization to the triad by the "T" system, somehow effected the release of Ca^{2+} from the lateral sacs of the sarcoplasmic reticulum to initiate contraction. It seemed reasonable to assume that calcium was released from the sarcoplasmic reticulum to initiate contractions, because studies on its relaxing properties had indicated that the sarcoplasmic reticulum was a storage site for calcium. Another important reason for this postulate was that the location of the lateral sacs of the sarcoplasmic reticulum with respect to the "T" system and the myofilaments, was especially suitable to rapidly link the two processes of excitation and contraction.

Winegrad's (1965, 1968) autoradiographic studies of the distribution and translocation of Ca^{45} during rest and

contraction of frog muscle fibres, provided evidence for the postulated role of the sarcoplasmic reticulum in initiating contraction. He found that during contraction, there was a movement of ^{45}Ca from the centre of the I band, where the lateral sacs of the sarcoplasmic reticulum are located, towards the A band.

The ability of the sarcoplasmic reticulum to regulate the level of Ca^{2+} in the myoplasm of a skeletal muscle fibre, appears to determine whether a muscle will be in a contracted or a relaxed state. There are several problems and implications associated with this function. It is not well understood how depolarization of the "T" system causes a release of calcium from the terminal sacs of the sarcoplasmic reticulum. Peachey (1965) on the basis of his examination of the morphology of the triad zone, suggested that depolarization of the "T" system could be transmitted to the terminal sacs, to increase their permability and cause Ca^{2+} release. However if depolarization causes release of calcium, it is difficult to explain how relaxation occurs under a maintained depolarization (Hodgkin and Horowitz 1960). Another problem encountered is the extreme difficulty of accurately determining calcium movements in an intact muscle. Therefore it remains uncertain whether the amount and the speed with which calcium is released and taken up by the sarcoplasmic reticulum in vivo, can adequately account for the contraction and relaxation respectively observed.

The functional significance of the sarcoplasmic reticulum becomes apparent after comparing different types of muscles. Peachey (1959, 1962) observed that in general "fast" acting striated muscle fibres had a well developed sarcoplasmic reticulum and "T" system, whereas in "slow" striated and smooth muscle fibres, the internal membrane system was considerably less developed. This structural-functional relationship which has been observed, provides a practical illustration which further suggests that the sarcoplasmic reticulum is especially well suited to rapidly couple excitation to contraction, by releasing an internal store of calcium ions to the region of the contractile elements. The rapid relaxation of muscles with well developed internal membrane systems, can be accounted for by the rapid removal of Ca^{2+} from the vicinity of the contractile proteins by the sarcoplasmic reticulum. The slow mechanical responses of muscles with poorly developed internal membrane systems, suggests that inward and outward diffusion of Ca^{2+} may be important in the processes of contraction and relaxation.

B) Mechanism of Calcium Accumulation by Sarcoplasmic Reticulum

During the past ten years investigators have attempted to elucidate the mechanism by which the sarcoplasmic reticulum accumulates calcium. The approaches used have mainly involved an examination of various properties of isolated fragmented sarcoplasmic reticulum.

Ebashi and Lipmann (1962) and Hasselbach and Makinose

(1962) observed that the ability of the sarcoplasmic reticulum to accumulate calcium, was greatly enhanced in the presence of ATP. There have been two main schools of thought concerning the role of ATP in this calcium uptake process. Ebashi and other investigators have proposed that ATP binding to sarcoplasmic membranes facilitates calcium binding to specific sites on the membrane. On the other hand, Hasselbach and co-workers have postulated that calcium uptake is an active transport process, which derives its energy from the hydrolysis of ATP. In order to examine the relative importance of binding and transport, Ebashi (1965) attempted to determine the amount of calcium taken up by the sarcoplasmic reticulum which existed in a bound and free state.

Ebashi (1961) on the basis of his studies with the "relaxing" factor and various chelating agents, suggested that the relaxation process in skeletal muscle was due to binding of Ca^{2+} to the relaxing factor. In an electron microscopic examination of sarcoplasmic reticulum vesicles which had accumulated Ca^{2+} from an oxalate containing medium, Hasselbach (1964a) observed that calcium oxalate was precipitated in the interior of the vesicles. In contrast to Ebashi's views these findings suggested that at least part of the calcium taken up by the sarcoplasmic reticulum, had crossed into the lumen of the vesicles as free ions and was deposited as calcium oxalate. By determining the minimum

effective concentration of oxalate for an enhanced calcium uptake effect, Ebashi estimated that only 10% of the calcium accumulated existed as free ions, and that the rest was in a bound form.

In another study Onishi and Ebashi (1964), using a rapid mixing technique, determined the velocity of Ca^{2+} uptake by sarcoplasmic reticulum vesicles. They speculated that this uptake was too rapid (60 μ Moles Ca^{2+} /min/mg protein) to be accounted for by a transport mechanism, but could be best explained by adsorption of Ca^{2+} to the outer surface of the vesicles. Weber (1966 a,b) however, questioned the reliability of this measurement, since it was considerably higher than values reported by a number of investigators whose preparations had the same level of steady state accumulation of calcium.

Carvahlo (1966) determined that sites were present in sarcoplasmic reticulum fragments which could electrostatically bind the physiological cations, that is Ca^{2+}, Mg^{2+}, K^{+} and H^{+}. Furthermore Carvahlo and Leo (1967) showed that these ions competed for available binding sites, and that ATP greatly enhanced the ability of Ca^{2+} to compete for these sites. Carvahlo estimated that at least 80% of the total Ca^{2+} accumulated was in a bound state, and could not be released by caffeine.

Two possible mechanisms were suggested by Carvahlo and Leo (1967) to account for the ability of ATP to promote

exchange of Ca^{2+} for bound Mg^{2+}, K^{+} and H^{+}. One possibility was that ATP directly promoted this exchange, by selectively increasing the affinity of surface binding sites for Ca^{2+}. Ebashi and Endo (1968) suggested that ATP might induce a conformational change in the binding site, which would result in a higher affinity for Ca^{2+}. Landgraf and Inesi (1969) provided evidence for a conformational change, since they observed a modification of the electron paramagnetic resonance (EPR) spectrum of iodacetamide-labelled sarcoplasmic reticulum upon ATP addition. Although ATP may induce a conformational change of the sarcoplasmic reticulum, one can not necessarily conclude that this will selectively increase the affinity of binding sites for Ca^{2+}. Furthermore it is not known whether Ebashi's (1968) postulated mode of action of ATP, depends merely on the binding of ATP or its hydrolysis.

Carvahlo and Leo (1967) suggested that a second possible role of ATP in facilitating Ca^{2+} binding, would be to provide an energy source for the transport of Ca^{2+} into the vesicles of sarcoplasmic reticulum. This transported Ca^{2+} could then bind passively to internal binding sites in exchange for Mg^{2+}, K^{+} and H^{+}. The suggestions that ATP could function indirectly to promote calcium binding seemed plausible, because Carvahlo's and Leo's experimental design could not distinguish between internal and external binding sites.

The evidence presented by various investigators has illustrated that a large portion of calcium which is accumulated by the sarcoplasmic reticulum is retained in some form of bound state. One can not conclude from this information whether calcium binding to the surface of the vesicular membrane, or calcium transport into the vesicle lumen and subsequent binding to internal sites account for calcium uptake.

Hasselbach's observations (1964a) of a calcium oxalate precipitate confined to the interior of sarcoplasmic reticulum vesicles, first established that at least part of the calcium accumulated was transported through the vesicular membrane. Furthermore his observations demonstrated that calcium and oxalate were transported against an activity gradient. These ions were transported from a level of ionic activity below the solubility product of calcium oxalate in the medium, to a level of ionic activity above the solubility product of calcium oxalate in the interior of the vesicles. Hasselbach concluded that calcium was the actively transported ion, since oxalate was not accumulated without calcium, whereas calcium was taken up without oxalate. In addition precipitation of calcium phosphate in the vesicles was observed when phosphate was substituted for oxalate.

Investigation of the ATPase properties of isolated sarcoplasmic reticulum by Hasselbach and Makinose (1962), provided information on the energy requirements for calcium

transport. They observed that in the absence of Ca^{2+}, the sarcoplasmic reticulum ATPase split ATP at a low rate ("basal" ATPase), and that this ATPase activity was not inhibited by the mercurial diuretic agent salyrgan. Upon the addition of calcium the rate of ATP hydrolysis increased 7-8 fold ("Calcium activated" ATPase). Furthermore this high rate of ATP hydrolysis was maintained until calcium uptake ceased.

Under most conditions a constant stoichiometry between Ca^{2+} uptake and Ca^{2+} ATPase was observed, such that 2 moles of Ca^{2+} were taken up for each mole of ATP hydrolyzed by the "Ca^{2+} activated" ATPase. (Hasselbach and Makinose 1963, Weber et al 1966b). This relationship was maintained even when the concentrations of ATP, Mg^{2+} and Ca^{2+} were altered, or various inhibitors (i.e. salyrgan) of both processes were used (Hasselbach and Makinose 1962, Hasselbach 1964b, and Martinosi 1964).

The findings of Hasselbach and other investigators seem to indicate that in most instances calcium uptake and "Ca^{2+} activated" ATPase are well correlated. This suggests that the energy derived from the hydrolysis of ATP by "calcium activated" ATPase, may be used for transporting calcium through the vesicular membranes.

The molecular mechanism by which ATP hydrolysis leads to calcium translocation into sarcoplasmic reticulum vesicles is not clear. Hasselbach and Makinose (1962) observed that

in addition to its activating effect on ATPase, Ca^{2+} stimulated an exchange of phosphate from ATP to ADP. Furthermore they found that Ca^{2+} uptake, "Ca^{2+} activated" ATPase and phosphate exchange were well correlated. In order to account for the coupling of these three processes, Hasselbach (1964b) proposed that active transport of Ca^{2+} occurred through the mediation of carrier molecules located in the vesicular membrane.

Recently several investigators have provided evidence for the formation of a phospho-membrane complex by isolated sarcoplasmic reticulum, which may be implicated as a carrier for transporting calcium (Yamamoto and Tonomura 1967, Makinose 1969, Martinosi 1969 and Inesi et al 1970). This complex was found to be acid stable, and its formation occurred through the incorporation of the terminal phosphate of ATP. The complex could be detected by incubating the sarcoplasmic reticulum with ^{32}P-ATP labelled in the terminal position.

These investigators observed that Ca^{2+} was essential for the formation of the phospho-membrane complex. In addition, Inesi et al (1970) showed that the ATPase and phospho-membrane complex formation were activated by the identical concentration of Ca^{2+}. These results strongly suggested that the phospho-membrane complex is an intermediate in the Ca^{2+} - dependent ATPase reaction.

Martinosi (1969) and Inesi et al (1970) observed that

Mg^{2+} had a dual role in the Ca^{2+} - dependent ATPase reaction. Mg^{2+} was not essential for phospho-membrane complex formation, but in its presence the steady-state level of the intermediate increased. Mg^{2+} also was important in the turnover of the phospho-membrane complex, since it accelerated the dephosphorylation of the intermediate.

Although the phospho-membrane complex appears to be directly involved in the Ca^{2+} - dependent ATPase reaction, its function in the calcium transport process is not clear. Direct involvement of the intermediate for transporting calcium would be implicated if the ATPase enzyme and transport system are one and the same. A recent approach used to examine the transport properties of the ATPase enzyme has involved the purification and characterization of the ATPase from sarcoplasmic reticulum (MacLennan 1970). The purified ATPase retained the same biochemical properties in isolation as in sarcoplasmic reticulum, and there was a six-fold increase in ATPase activity. MacLennan et al (1971) observed that removal of deoxycholate which has been used to disperse the enzyme, caused the enzyme to spontaneously form vesicular membranes. These re-formed membranes had all the properties of sarcoplasmic reticulum which were necessary for Ca^{2+} transport, but their calcium accumulating ability was low.

These findings suggested that the ATPase was the transport enzyme, but the reformed membranes were not sufficiently

closed to permit calcium accumulation within. However, MacLennan et al (1971) could not rule out the possibility that Ca^{2+} transport proceeds through the mediation of separate carriers which only derive energy from the ATPase.

There seems to be sufficient evidence to conclude that a portion of the Ca^{2+} accumulated by the sarcoplasmic reticulum is transported into the vesicles. A major problem to be considered is the manner in which Ca^{2+} is sequestered. Recently MacLennan and Wong (1971) have isolated the protein "Calsequestrin", which was hydrophobically bonded in the interior of the membranes of sarcoplasmic vesicles. On the basis of its calcium binding properties they postulated that calsequestrin is a major site of calcium sequestration in the interior of sarcoplasmic reticulum membranes, and that calcium binding to this protein could account for a large proportion of the Ca^{2+} accumulated.

The experimental evidence cited, indicates that most of the calcium which is transported into the sarcoplasmic reticulum is bound to an internal site, while only a small amount is in the free form. In addition it seems likely that most of the calcium accumulated is transported into the sarcoplasmic reticulum vesicles. Nevertheless, one can not at this time eliminate the possibility that calcium adsorption onto external binding sites plays a role in calcium accumulation.

C) Objectives of Study

The objectives of this study were to examine the ATPase activity and Ca^{2+} uptake processes of isolated fragmented sarcoplasmic reticulum obtained from rabbit skeletal muscle. Since the role of the "Ca^{2+} activated" ATPase enzyme in the accumulation of Ca^{2+} by the sarcoplasmic reticulum has not yet been clearly defined, experiments were conducted in order to further elucidate the nature of the relationship of these two processes. In particular, the degree of parallelism between Ca^{2+} uptake and "Ca^{2+} activated" ATPase was examined. The experimental approaches which were used included studies of both the effects of chemical agents, as well as the physical effects of temperature on Ca^{2+} uptake and "Ca^{2+} activated" ATPase. The rationale of this type of approach was that if these agents or conditions affected Ca^{2+} uptake or "Ca^{2+} activated" ATPase in a similar manner, such findings may indicate that these processes are closely related. On the other hand, if only one process is affected, this could indicate that Ca^{2+} uptake may be dissociated from "Ca^{2+} activated" ATPase.

CHAPTER II

METHODS AND MATERIALS

Tissue Preparation

Male New Zealand white rabbits weighing 2.0 - 2.5 Kg., were killed by a sharp blow to the back of the head. The back muscles were excised, and fat and connective tissue were dissected away. These muscles were then chilled in a .9% NaCl solution. Approximately 100 grams of muscle were used for the preparation of the sarcoplasmic reticulum fractions.

Homogenization

All subsequent steps were carried out in the cold room at 4°C. The muscle was cut into small pieces and 7 ml. of homogenizing solution added for each gram of tissue. Initially the composition of the homogenizing solution was .1 M KCl, 50 mM glycyl-glycine and 50 mM histidine (pH 7.2). This was similar to the solution used by Martinosi and Feretos (1964a), with the modification of an increase in buffer concentrations from 5 mM to 50 mM in order to process a greater quantity of tissue in a single operation. In the later experiments this solution was replaced by 20 mM histidine pH 7.2 which was found to provide adequate buffer capacity. The muscle and homogenizing medium were blended in an "Osterizer Pulse Matic" for 30 seconds. This procedure was repeated 3 times with a two minute interval between each blending for cooling.

Differential Centrifugation

The sarcoplasmic reticulum fraction was isolated from the homogenate by differential centrifugation according to

the method described by Martinosi and Feretos (1964a). All the centrifugations were performed in a Sorvall RC - 2B refrigerated centrifuge utilizing the SS - 34 angle head. After each centrifugation the pH of the solution was readjusted to 7.2 with Tris when required. Initially the homogenate was centrifuged at 1000 x g for twenty minutes for the removal of myofibrils. The supernatant was passed through 4 layers of gauze for removal of any remaining connective tissue, and the filtrate centrifuged at 8000 x g for twenty minutes. This procedure sedimented a so-called mitochondrial pellet which was discarded, and the remaining supernatant was centrifuged at 25,000 x g for one hour. The pellet which was obtained from the 25,000 x g centrifugation was identified as the fragmented sarcoplasmic reticulum fraction. The pellet was resuspended by hand homogenization with a Tenbroeck tissue grinder in a solution containing 50mM KCl, 5mM $MgCl_2$, 25 mM histidine and 25 mM glycyl-glycine at pH 7.2. This suspension was diluted to a protein concentration of about 4 mg./ml. 1 ml aliquots were stored at -15°C for use in subsequent experiments. In addition some of the pellets which contained the sarcoplasmic reticulum fraction were fixed by a phosphate-buffered 5% glutaraldehyde solution for electron microscopic studies.

Protein Determination

The protein nitrogen content of a .1 ml aliquot sarcoplasmic reticulum was determined by the method of Lowry

et al (1951). The standard reference curve was prepared by using a solution of crystalline bovine albumin, and the optical densities were read at 650 mμ on a Beckman DB-G grating spectrophotometer. In most experiments the protein nitrogen content of the sarcoplasmic reticulum fraction was adjusted to 400 μg/ml.

Assay Procedures

During the course of this investigation the calcium accumulating and ATPase properties of isolated sarcoplasmic reticulum were studied. Simultaneous measurement of both these processes was facilitated by using a millipore filtration apparatus. The incubation solution contained 50 mM KCl, 5 mM Tris base and .25 mM EGTA. Variable amounts of $CaCl_2$, potassium oxalate, ATP and $MgCl_2$ (final concentrations of ATP and $MgCl_2$ were equal) were added to the incubation solution for different experiments. ^{45}Ca was the last reagent added to the incubation solution, in experiments where calcium uptake was measured.

(a) Determination of ATPase Activity

Incubation was carried out in a gently shaking Dubnoff water bath. Disodium ATP (Sigma) was used as the substrate, and the reaction was initiated by the addition of .1 ml of sarcoplasmic reticulum. All the reactants were first brought to incubation temperature. Initially when only ATPase activities were measured, the reaction was terminated by the addition of 1.5 ml. of cold 5% TCA to the reaction vessel.

The precipitated protein was filtered on a 7 cm. Whatman #50 hardened filter paper. In the majority of experiments where both Ca^{2+} uptake and ATPase activities were determined, the reaction was terminated without the addition of acid by the rapid separation of enzyme protein from the reaction medium, by filtration through a millipore filter. The sarcoplasmic reticulum was retained on millipore filters of pore size .45 μ. The protein-free filtrates obtained from either method were chilled and retained for inorganic phosphate analysis.

The inorganic phosphate in a 2 ml aliquot of protein free filtrate was determined colorimetrically by the method of Fiske and Subba Row (1925) as modified by Aldridge (1962), and using amidol as the reducing agent (Skou 1957). Corrections were made for non-enzymatic hydrolysis of ATP.

ATPase activity was expressed as μ moles inorganic phosphate liberated from ATP/mg of enzyme protein Nitrogen. The ATPase activity apparent in the absence of calcium is referred to as "basal" ATPase. The ATPase activity in the presence of added calcium is defined as the "total" ATPase, and the difference between the total ATPase and "basal" ATPase is the "Ca^{2+} activated" ATPase.

(b) Determination of Calcium Uptake

A small amount of ^{45}Ca with a specific activity of 2-10 mCi/mg. Ca^{2+} was added to an appropriate incubation solution. This ^{45}Ca-containing solution was then incubated

with sarcoplasmic reticulum, and after a certain time interval the reaction was stopped by filtration through a millipore filter. By means of this procedure, the sarcoplasmic reticulum with its accumulated calcium was trapped on the filter pad. The non-specifically bound calcium on the millipore filter, was reduced to a constant "blank" value by the addition of two 3.5 ml. aliquots of washing solution. The washing and incubation solutions were similar except for the absence of ^{45}Ca and ATP in the washing solution. After this washing procedure, the millipore filters were placed in counting vials, and 10 ml. of a 0.5% 2,5 diphenyloxazole (PPO) and 5% Beckman's Biosolve 3 (BBS-3) in toluene fluor were added to the vials. The radioactivity was counted in a Beckman LS-100 liquid scintillation counter.

In order to calculate the amount of calcium taken up by the sarcoplasmic reticulum, the following procedure was used. A 0.1 ml. aliquot of incubation solution was counted in the same fluor used for the millipore filters. The activity of incubation solution was expressed as cpm/ml. Since the total amount of added calcium in 1 ml. of incubation solution is known, this activity can be expressed as cpm/μ Mole Calcium. An assumption which is made for this calculation is that any contaminant Ca^{2+} is very small in comparison to the amount added, and that the ^{45}Ca radioactivity is evenly distributed over all the total

calcium in the incubation solution.

The millipore filters containing sarcoplasmic reticulum and its accumulated calcium were then counted, and their activities were expressed as raw counts (cpm). Corrections were made for counts adsorbed onto the millipore filter per se, and the corrected count values were converted into μ Moles Calcium, for calculation of the calcium taken up by the sarcoplasmic reticulum. It was not necessary to make any corrections for quenching because the incubation solutions and the millipore filters were counted at the same efficiency level. The external standard ratio value for each sample provided a measure of the counting efficiency.

It should be noted that the value of calcium taken up by the sarcoplasmic reticulum is an approximate one. This is because the amount of contaminant calcium present in the incubation solution is not known accurately but is less than 10^{-6}M. The calcium uptake which was determined in the various experiments was expressed as μ Moles Ca^{2+} uptake/mg. enzyme protein Nitrogen.

CHAPTER III

GENERAL Ca^{2+} UPTAKE AND ATPase PROPERTIES OF SARCOPLASMIC RETICULUM

Results

Preliminary studies were carried out to define the experimental conditions necessary to study both the ATPase and Ca^{2+} uptake processes of fragmented sarcoplasmic reticulum. Initially the "basal" ATPase and "Ca^{2+} activated" ATPase properties of sarcoplasmic reticulum as defined by Hasselbach and Makinose (1962) were examined.

The "basal" ATPase activity was measured in the absence of added Ca^{2+}. EGTA which is an effective as well as a specific complexing agent for Ca^{2+}, was added to the incubation solution in order to complex any contaminant Ca^{2+} in the reaction system. Incubation solutions (described in "Methods") with variable concentrations of EGTA, and 5 mM ATP and 5 mM $MgCl_2$ were incubated with 40 µg. of sarcoplasmic reticulum for five minutes. Different levels of ATP hydrolysis as determined by inorganic phosphate release, were observed at various EGTA concentrations (Table 1). ATP hydrolysis decreased as the EGTA concentration of the incubation solution was increased. For practical reasons it was decided that for subsequent experiments the amount of ATP hydrolyzed by sarcoplasmic reticulum in a .25 mM EGTA Ca^{2+} "free" incubation solution, would be defined as the "basal" ATPase activity.

In order to determine the "Ca^{2+} activated" ATPase activity of sarcoplasmic reticulum it is necessary to measure ATP hydrolysis in the absence and presence of

calcium. Variable amounts of $CaCl_2$ were therefore added to the .25 mM EGTA incubation solution which was used to measure "basal" ATPase activity. Initially the rate of ATP hydrolysis was elevated by increasing the amount of $CaCl_2$ added to the system. A plateau was reached at a concentration of $CaCl_2$ between .30 mM and .40 mM. Further addition of $CaCl_2$ resulted in a decrease in ATP hydrolysis. (Table II). Therefore an incubation solution containing both .35 mM $CaCl_2$ and .25 mM EGTA was used for future studies of the "Ca^{2+} activated" ATPase properties of sarcoplasmic reticulum as these conditions were optimal.

Since most studies involved a measurement of the Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities of a single sample of sarcoplasmic reticulum, the same incubation solution was used for the investigation of both of these parameters. As it was considered undesirable to terminate the reaction by acid denaturation or by prolonged centrifugation, either of which could lead to changes in the amount or nature of the calcium binding properties of the preparation, millipore filtration was explored as a possible method of separating the sarcoplasmic reticulum from its reaction medium at the pH of the experiments. Millipore filters with pore diameters from 0.3 μ to 3.0 μ were tested and a pore size of 0.45 μ was selected since this permitted filtration within two seconds and retained 95% of the total protein of the system.

TABLE I

Effects of EGTA on ATPase activity

EGTA Conc. mMoles/l	ATPase Activity μMoles P_i/mg protein N
0	7.74
.0625	7.44
.125	6.84
.25	6.26
.50	6.26
1.00	6.12

Sarcoplasmic reticulum samples were incubated for 5 minutes at 37°C.

Assay Media: 5 mM $MgCl_2$, 5 mM ATP, 50 mM KCl, 5 mM Tris, with variable concentrations of EGTA.

Values shown are the average of triplicate assays.

TABLE II

Effects of $CaCl_2$ on "Ca^{2+} activated" ATPase activity

$CaCl_2$ Conc. mMoles/l	"Ca^{2+} activated" ATPase µMoles P_i/mg protein N
0	0
.20	.88
.30	7.96
.40	7.96
.50	6.44
.60	5.34
1.00	2.62

Sarcoplasmic reticulum samples were incubated for 5 minutes at 37°C.

Assay Media: 5 mM $MgCl_2$, 5 mM ATP, 50 mM KCl, 5 mM Tris, .25 mM EGTA and variable concentrations of $CaCl_2$.

Values shown are the average of duplicate assays.

In order to compare the properties of our sarcoplasmic reticulum preparation with those reported by other investigators, the following experimental parameters were investigated.

A) Electron Microsopic Appearance

Electron micrographs of pelleted sarcoplasmic reticulum were prepared by Mr. Gus Duchon of the Pharmacology Department. An example of an electron micrograph is shown in Figure 1 where it can be seen that the predominant structure is vesicular elements. Most of these elements are bounded by a well defined continuous membrane. The small particles seen in this and other micrographs may be glycogen particles (Ebashi and Lipmann 1962). Another noteworthy feature is that there appears to be very little contamination of our preparations with nuclei, myofilaments or mitochondria. These electron microscopic studies suggest that the morphology of our preparation is similar to the sarcoplasmic reticulum preparations described by other investigators (Ebashi and Lipmann 1962, Hasselbach and Makinose 1963, and Martinosi 1964).

B) Effects of Oxalate

Hasselbach and Makinose (1961, 1962, 1963) observed that oxalate markedly increased the amount of calcium accumulated by the sarcoplasmic reticulum. In order to test the effects of oxalate, a study was made of Ca^{2+} uptake over five minutes at 0, 1, 3 and 5 mM potassium

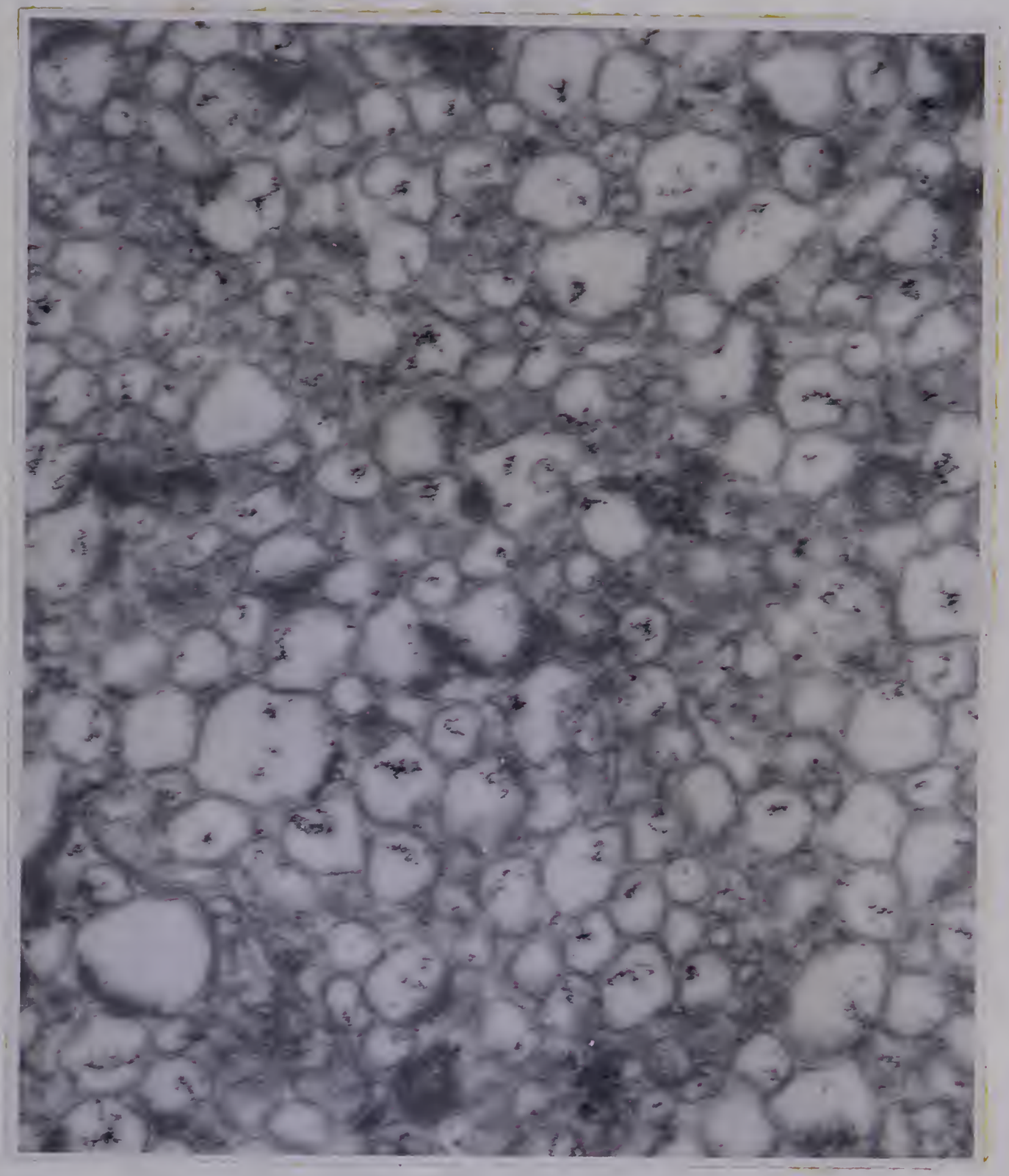

Figure 1: An electron micrograph of the sarcoplasmic reticulum fraction obtained from rabbit skeletal muscle. Sarcoplasmic reticulum fraction was pelleted after centrifugation at 25,000 x g. Magnification 60,000 x.

oxalate concentrations. A 20-fold increase in Ca^{2+} uptake was observed upon the addition of 1 mM oxalate, and this was further doubled by increasing the oxalate concentration to 5 mM (Table III).

When the oxalate concentration was elevated from 1 mM to 5 mM, the "Ca^{2+} activated" ATPase activity was increased 1.7 fold. The "basal" ATPase activity does not appear to be affected when the oxalate concentration is increased. In the remaining experiments incubation solutions containing 3 mM potassium oxalate were used for the study of the calcium uptake and ATPase processes.

Typical plots of the time dependence of Ca^{2+} uptake and ATPase activities in the presence of 3 mM oxalate, are illustrated in Figures 2 and 3. Calcium uptake increases linearly up to one minute. After the first minute the rate of calcium uptake declines, and it appears that a steady state level of calcium accumulation is reached at about five minutes. In contrast the "Ca^{2+} activated" ATPase hydrolyzes ATP at a constant rate for the first three minutes, and the rate of this hydrolysis is increased slightly during the third to fifth minute of incubation. The "Ca^{2+} activated" ATPase hydrolyzes ATP at a rate 5-6 times faster than the "basal" ATPase.

These time dependent studies have important implications for the examination of the quantitative relationship, of the ratio of Ca^{2+} accumulated to ATP split

TABLE III

Effects of oxalate on Ca^{2+} uptake and ATPase activities

Oxalate Conc. mM/l	Ca^{2+} uptake µMoles Ca^{2+}/mg protein N	Ca^{2+} ATPase µMoles P_i/mg protein N	*basal ATPase µMoles P_i/mg protein N
0	.12	3.34	1.10
1	2.30	3.70	1.30
3	4.00	4.44	1.10
5	4.68	6.28	1.10

Sarcoplasmic reticulum samples were incubated for 1 minute at 37°C.

Assay media: 5 mM $MgCl_2$, 5 mM ATP, variable concentrations of oxalate, .35 mM $CaCl_2$, tracer ^{45}Ca, .25 mM EGTA, 50 mM KCl, 5 mM Tris.

* $CaCl_2$ was omitted for basal ATPase determinations.

Values shown are the average of duplicate assays.

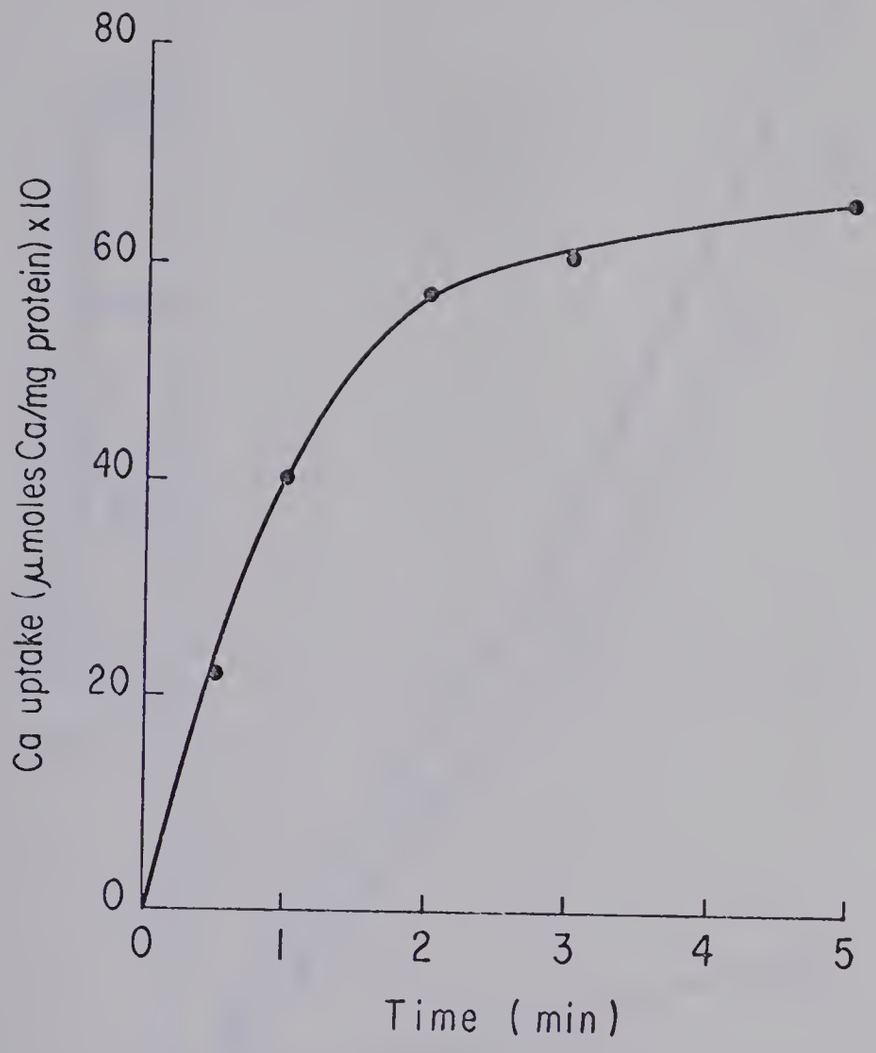

Figure 2: A typical plot of the time-dependence of Ca^{2+} uptake. Sarcoplasmic reticulum was incubated at 37°C in an assay medium containing 3mM oxalate, 5mM ATP and 5mM $MgCl_2$, and other substituents described in TABLE IV. Assays were in duplicate.

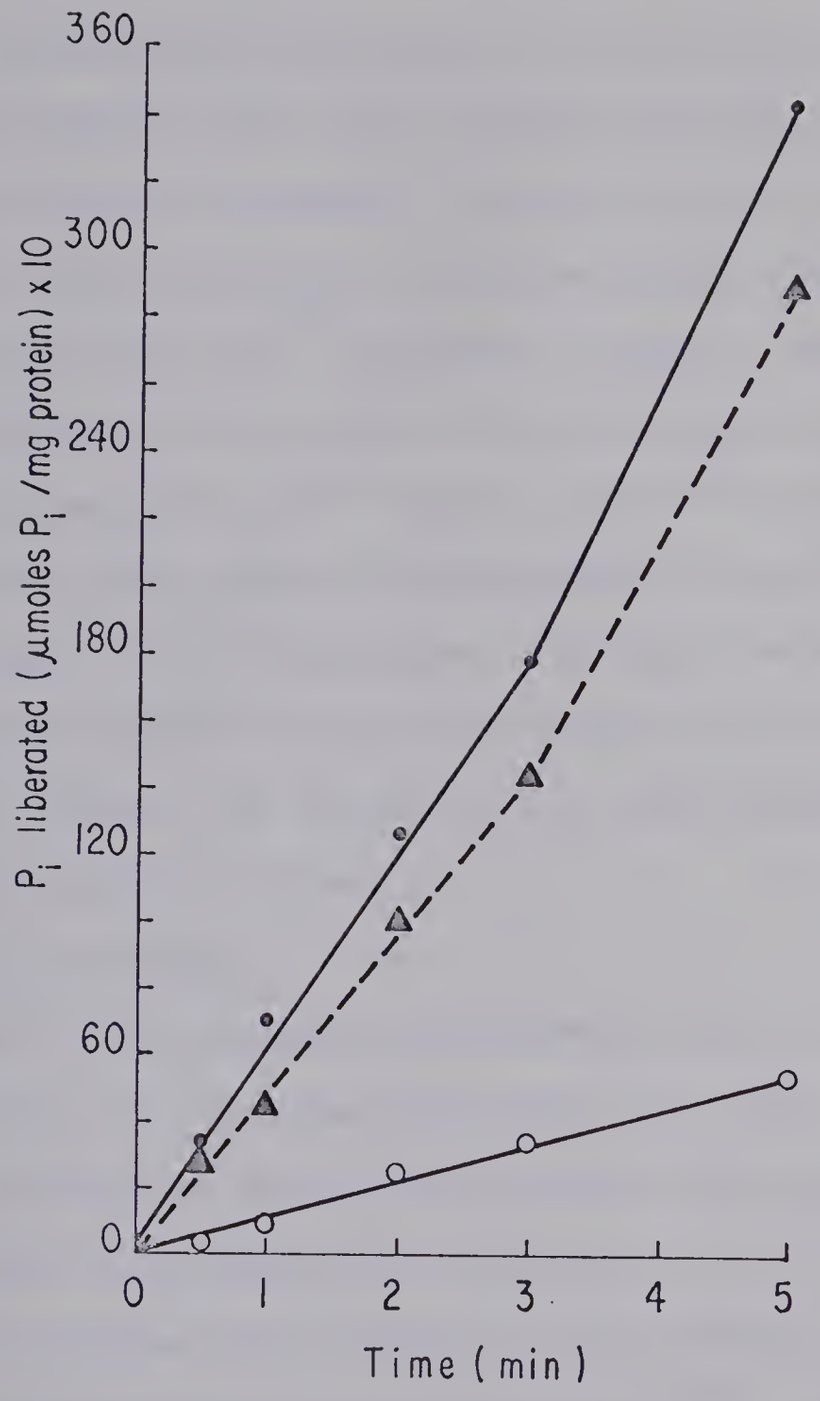

Figure 3: A typical plot of the time-dependence of ATPase activities. Sarcoplasmic reticulum was incubated at 37°C in an assay medium containing 3 mM oxalate, 5 mM ATP and 5 mM $MgCl_2$ and other substituents described in TABLE IV. (○ - ○) Total ATPase activity, (△ -- △) "Ca^{2+} activated" ATPase activity, (○ - ○) "basal" ATPase activity. Assays were in duplicate.

by the "Ca^{2+} activated" ATPase. Since the rate of Ca^{2+} uptake declines much more rapidly than the rate of "Ca^{2+} activated" ATPase, this ratio becomes smaller as the incubation time is increased. Usually a one minute incubation time was chosen for quantitative comparisons of Ca^{2+} uptake and "Ca^{2+} activated" ATPase. The reason for this choice was that the one minute point is on the linear portion of the Ca^{2+} uptake and "Ca^{2+} activated" ATPase versus time plots, and therefore at one minute the initial rates of both processes are approximated. The relationship of Ca^{2+} uptake to ATP split by the "Ca^{2+} activated" ATPase, was about 1:1 at 3 mM oxalate after a one minute incubation time.

C) Effects of Ageing

One of the problems encountered in the investigation of sarcoplasmic reticulum properties, was that the amount of calcium taken up by the sarcoplasmic reticulum declined rapidly when the preparation was stored at 4°C. In an effort to overcome this problem, 1 ml. aliquots of undiluted sarcoplasmic reticulum were frozen at -15°C. immediately after preparation. Samples were thawed at room temperature before each experiment. The effectiveness of this procedure was tested, by comparing the Ca^{2+} uptake and ATPase activities of sarcoplasmic reticulum which was stored at 4°C and -15°C (Table IV). The activities are expressed for one minute incubations, but the effects of

ageing are similar throughout the three minute incubation period.

Calcium uptake by sarcoplasmic reticulum which was stored at 4°C was almost completely abolished after eight days. In contrast preparations which were stored at -15°C had their calcium uptake reduced by approximately 20% after twenty days ageing. It is of interest to note that the initial Ca^{2+} uptake after one day of storage at -15°C was almost twice that of sarcoplasmic reticulum stored at 4°C. The "Ca^{2+} activated" ATPase activities of sarcoplasmic reticulum stored under both conditions increased by approximately the same amounts with ageing, whereas "basal" ATPase activities were fairly consistent. The findings for sarcoplasmic reticulum stored at 4°C, illustrate that even though Ca^{2+} uptake is almost completely abolished after ageing the "Ca^{2+} activated" ATPase activity is maintained and appears to have increased. This finding suggests that Ca^{2+} uptake may be capable of dissociation from "Ca^{2+} activated" ATPase. In contrast the sarcoplasmic reticulum which is stored at -15°C is able to continue accumulating Ca^{2+} for a suitable time period, and therefore this method of storage was used in the remaining experiments.

D) Effects Due to Variations of ATP Concentration

Calcium uptake and "Ca^{2+} activated" ATPase activities are recorded at different concentrations of ATP in Table V. It is apparent that the addition of ATP to the incubation

TABLE IV

Effects of ageing on Ca^{2+} uptake and ATPase activities

Days Aged	Ca^{2+} uptake		Ca^{2+} ATPase		basal ATPase	
	µMoles Ca^{2+}/mg protein N		µMoles P_i/mg protein N			
	4°C	-15°C	4°C	-15°C	4°C	-15°C
1	.82	1.52	3.34	2.92	1.66	1.66
5	.52	1.42	3.48	3.30	2.02	1.66
8	.06	1.16	4.44	3.76	1.66	2.02
20	---	1.20	----	4.18	----	1.66

Sarcoplasmic reticulum samples were incubated for 1 minute at 37°C.

Assay Media: 5 mM ATP, 5 mM $MgCl_2$, 3 mM oxalate, .35 mM $CaCl_2$, tracer ^{45}Ca, .25 mM EGTA, 50 mM KCl, and 5 mM Tris.

4°C and -15°C are temperatures of which preparations were stored prior to assay.

Values shown are average of assays in duplicate, --- indicates assays not done.

TABLE V

Effects of ATP on Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities measured at two different incubation times

ATP Conc. mMoles/l	Ca^{2+} uptake µMoles Ca^{2+}/mg protein N		"Ca^{2+} activated ATPase µMoles P_i/mg protein N	
	(a)	(b)	(a)	(b)
0	.24	.26	0	0
2.5	3.82	6.86	4.18	8.62
5.0	3.98	7.06	4.22	12.16

Sarcoplasmic reticulum samples incubated for 1 minute (a) and 3 minutes (b) at 37°C.

Assay media: variable concentrations of ATP and $MgCl_2$ (ATP = $MgCl_2$) and other substituents described in TABLE IV

Values shown are the average obtained from assays at least in duplicate.

solution greatly enhances Ca^{2+} uptake. Concentrations of 2.5 mM and 5 mM ATP appear to be equally effective for supporting Ca^{2+} uptake. The "Ca^{2+} activated" ATPase activities after a one minute incubation are the same at 2.5 mM and 5 mM ATP. After three minutes incubation, however; the "Ca^{2+} activated" ATPase activity at 2.5 mM ATP was approximately 33% less than at 5 mM ATP. The calcium uptake and "Ca^{2+} activated" ATPase activities after one minute incubations are unaltered, when the ATP concentration is increased from 2.5 mM to 5 mM. Since a one minute incubation time was chosen for comparison of Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities in most experiments, 2.5 mM ATP was often used in these experiments.

E) Effectiveness of Various Nucleoside Triphosphates

Several investigators have observed that Ca^{2+} uptake can be supported to varying degrees in the presence of nucleoside triphosphates (NTP's) other than ATP. (Martinosi and Feretos 1964a, Hasselbach 1964a). In addition they have observed that NTP's are hydrolyzed at a low rate in the absence of Ca^{2+}; and that the addition of Ca^{2+} increased the rate of NTP hydrolysis. On the basis of these findings, they have proposed that sarcoplasmic reticulum has a general "basal" NTPase which is active in the absence of Ca^{2+}, and a "Ca^{2+} activated" NTPase which becomes active upon the addition of Ca^{2+}.

In order to test our preparation for these properties,

TABLE VI

Effects of Nucleoside Triphosphates on Ca^{2+} uptake and "Ca^{2+} activated ATPase activities

NTP	Ca^{2+} uptake µMoles Ca^{2+}/mg protein N	"Ca^{2+} activated" ATPase µMoles P_i/mg protein N
ATP	2.28	3.10
ITP	1.94	7.74
CTP	1.50	6.40
UTP	.88	3.28
GTP	.78	1.04

Sarcoplasmic reticulum samples were incubated for 1 minute at 37°C.

Assay media:2.5 mM $MgCl_2$, 2.5 mM various NTP's and other substituents described in TABLE IV

Values shown are the average of duplicate assays in two separate experiments..

a study of the time dependence of Ca^{2+} uptake and NTPase activities was undertaken. The NTP's used were Guanosine Triphosphate (GTP), Cytidine Triphosphate (CTP), Uridine Triphosphate (UTP) and Inosine Triphosphate (ITP). For comparison ATP was used as a control in each experiment. Table VI indicates that all these NTP's could support Ca^{2+} uptake, and the order of effectiveness was ATP > ITP > CTP > UTP > GTP. These results are qualtitatively similar to Hasselbach's findings (1964b), except for the reversal of UTP and GTP.

Discussion

Sarcoplasmic reticulum was prepared from homogenized rabbit skeletal muscle by differential centrifugation, according to the procedure described in "Methods". When various parameters were investigated it was observed that the properties of our sarcoplasmic reticulum preparation were qualitatively similar to those reported by other investigators.

For example, closed vesicular elements are the predominant structures seen in electron micrographs of our preparation, and these also appear to be the main structures of sarcoplasmic reticulum preparations described by other investigators (Ebashi and Lipmann 1962, Hasselbach and Makinose 1963, and Martinosi 1964).

It was demonstrated that in the presence of Mg^{2+} and ATP, calcium ions were taken up by the sarcoplasmic

reticulum and that this uptake was greatly enhanced when oxalate was present in the reaction system (Table IV). This potentiating effect of oxalate has been explained on the basis that oxalate and other anions form salts of low solubility, thereby reducing the free Ca^{2+} concentration in the lumen of sarcoplasmic reticulum vesicles, and thus promoting Ca^{2+} transport until the precipitate fills all the available space (Ebashi and Endo 1968).

Hasselbach (1964a) had demonstrated that maximal Ca^{2+} uptake occured when 5 mM oxalate was present in the incubation solution. However, a concentration of 3 mM oxalate, at which there was less than maximal Ca^{2+} uptake, was chosen for our subsequent experiments so that an enhancement of Ca^{2+} uptake, could be detected if it occured.

In addition to the Ca^{2+} uptake properties it was also shown that our sarcoplasmic reticulum preparation exhibited ATPase properties (Figure 3) which were similar to the ones initially described by Hasselbach and Makinose (1962). For example, sarcoplasmic reticulum sustained a low rate of ATP hydrolysis (Figure 3) in the absence of Ca^{2+} ("basal" ATPase activity). When Ca^{2+} was added to the incubation solution, the rate of ATP splitting was increased. When 3 mM oxalate was present in the incubation, the "Ca^{2+} activated" ATPase hydrolyzed ATP at a rate of 5 - 6 times faster than the "basal" ATPase.

In our experiments the numerical relationship of Ca^{2+} uptake to ATP split by the "Ca^{2+} activated" ATPase was about 1:1, when these processes were measured in the presence of 3 mM oxalate and after a one minute incubation. This value is somewhat lower than the 2:1 ratio observed in the presence of oxalate by Hasselbach (1964b). Although there seems to be a discrepancy between our results and Hasselbach's, it is important to realize that the quantitative relationship between these two processes will depend on the experimental conditions which are used for the determination of both Ca^{2+} uptake and "Ca^{2+} activated" ATPase. For example, one must specify the concentration of oxalate present and the time of the incubation period particularly since Ca^{2+} uptake declines more rapidly than "Ca^{2+} activated" ATPase activity (Figures 2 and 3). Another important factor to be considered is the Ca^{2+} concentration of the incubation solution. A decrease in "Ca^{2+} activated" ATPase activity was observed when the total $CaCl_2$ concentration of the incubation solution was increased beyond .40 mM (Table II). When 3 mM oxalate was present in the incubation solution, similar increases of $CaCl_2$ concentration did not decrease Ca^{2+} uptake (Table X). Similar observations have been made by Martinosi and Feretos (1964b), when oxalate was present for the determination of both Ca^{2+} uptake and "Ca^{2+} activated" ATPase activity.

Possible contamination of our preparation with "Ca^{2+}

activated" ATPases not concerned with Ca^{2+} uptake, for example actomyosin ATPase, might also account for the lower ratio of Ca^{2+} taken up to ATP split by "Ca^{2+} activated" ATPase in our experiments. For example, by employing a KCl extraction procedure Martinosi (1968) removed an actomyosin-type ATPase from his sarcoplasmic reticulum fraction which accounted for about 20% of the total protein of the fraction. Since a KCl extraction procedure was not used for the isolation of our preparation, there is the possibility of actomyosin ATPase contamination.

Although the hydrolysis of ATP by sarcoplasmic reticulum is generally referred to as an "ATPase" activity it was originally described by Hasselbach (1964a) as a non-specific nucleotidase activity. When the substrate specificity of our preparation was studied we found that ATP, CTP, ITP, GTP and UTP could to varying degrees support Ca^{2+} uptake, and that the rate of NTP hydrolysis was increased upon the addition of Ca^{2+} (Table VI). On the basis of similar findings Hasselbach (1964a) postulated that a general non-specific "Ca^{2+} activated" NTPase provided the energy required for Ca^{2+} uptake by the hydrolysis of NTP. However, it has been reported by Makinose (1966) that there are nucleoside transphorylases associated with sarcoplasmic reticulum which are capable of transferring the terminal phosphates of NTP's such as

GTP, and ITP to ADP. Therefore if small amounts of adenine nucleosides are present as impurities in the other NTP's, the generation of ATP may be an important stimulus to calcium accumulation. The NTP's used in our experiments were not checked for purity, because this was not considered to be important for our studies.

It is of interest to consider the effects of ageing on Ca^{2+} uptake and "Ca^{2+} activated" ATPase activity (Table IV). The results obtained for the preparation which was stored at 4°C, illustrated that there is a suppression of Ca^{2+} uptake whereas "Ca^{2+} activated" ATPase is actually increased, as the time of storage is increased. These findings might appear to indicate that Ca^{2+} uptake of sarcoplasmic reticulum becomes dissociated from "Ca^{2+} activated" ATPase with the ageing of the preparation. However, a more probable explanation is suggested by the electron microscopic observations of Ebashi and Lipmann (1962) who observed that the membranes of the vesicular elements of an aged preparation were not sharply defined and were often discontinuous. In contrast the vesicular membranes of fresh preparations were well-defined and continuous.

Thus there seems to be an alteration of the structural integrity of the vesicular membranes when sarcoplasmic reticulum is allowed to age, and hence it is conceivable that there is a resultant increase in the

membrane permeability to Ca^{2+}. Therefore, even though net Ca^{2+} uptake is decreased upon ageing, there is not necessarily a decrease in the amount of Ca^{2+} transported. A possible explanation for the maintained Ca^{2+} accumulating ability of preparations stored at -15°C, is that freezing allows the membrane structural integrity to be preserved.

Alteration of the membrane structure may also account for the increase in "Ca^{2+} activated" ATPase observed with ageing of the preparation. This structural alteration could expose additional active sites, to allow for an increased hydrolysis of ATP. If this postulate is valid, it would follow that some of the active sites of the "Ca^{2+} activated" ATPase enzyme are not located on the external surface of the membrane.

Our experiments do not provide evidence to allow an assessment of either of these postulates.

CHAPTER IV

EFFECTS OF CHEMICAL AGENTS AND TEMPERATURE ON Ca^{2+} UPTAKE AND Ca^{2+} ACTIVATED ATPase

Results

A) Effects of Chemical Agents

Several chemical agents have been found to effect Ca^{2+} uptake and "Ca^{2+} activated" ATPase in a parallel manner (Hasselbach 1966). These findings suggest that the two processes are closely related, and that the energy derived from the hydrolysis of ATP by the "Ca^{2+} activated" ATPase of sarcoplasmic reticulum is utilized to accumulate Ca^{2+}. In contrast there have been a few agents such as oleic acid (Martinosi 1964) and diethyl ether (Inesi et al 1967) which have been reported to suppress Ca^{2+} uptake but not "Ca^{2+} activated" ATPase. In addition our data on the physical effects of ageing seem to demonstrate an uncoupling of Ca^{2+} uptake from "Ca^{2+} activated" ATPase. It was felt that additional studies, to observe whether various types of chemical agents effect Ca^{2+} uptake and "Ca^{2+} activated" ATPase in a parallel manner, could provide further information on the relation of these two processes. The agents used included the sulfhydryl reagents N-ethylmaleimide (NEM) and mersalyic acid (salyrgan), quinidine, ouabain and procaine.

Effects of Sulfhydryl Reagents

Hasselbach and Seraydarian (1966) demonstrated that Ca^{2+} uptake and "Ca^{2+} activated" ATPase activity were both completely suppressed when when about 65% of the available sulfhydryl groups of sarcoplasmic reticulum were substituted by salyrgan or NEM. Since both agents are able to inhibit

Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities, they were used to examine the relationship of the two processes.

(a) N-ethylmaleimide (NEM)

Freshly prepared NEM (pH 7.0) at different concentrations was preincubated with sarcoplasmic reticulum. A preliminary measure of the inhibiting effect of NEM was obtained by comparing the Ca^{2+} uptakes of an untreated control and a sarcoplasmic reticulum sample of which had been pretreated with this agent prior to assay. The amount of inhibition observed in the presence of NEM depended on the concentration of NEM used, and also on the time period for which it was preincubated with sarcoplasmic reticulum. For example a 94% inhibition of Ca^{2+} uptake was observed when 10^{-3}M NEM was preincubated with sarcoplasmic reticulum suspension (400 μ g Protein N/ml) for thirty-five minutes.

In order to examine the effects of NEM further, a study was made of Ca^{2+} uptake and ATPase activities over three minutes, in the presence and absence of NEM in the assay system. NEM was used under conditions which produced 94% inhibition of Ca^{2+} uptake. The general results are illustrated by the observations after a one minute incubation and indicate that NEM inhibits Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities to approximately the same degree. (Table VII). The basal ATPase appears to be increased in the presence of N-ethylmaleimide. However,

this may be misleading because of the low level of ATP hydrolysis which occurs under these conditions, and this result is thus subject to greater experimental error.

(b) Mersalyic acid (salyrgan)

Salyrgan, as in the case of NEM, was most effective in inhibiting Ca^{2+} uptake when it was preincubated with sarcoplasmic reticulum for thirty-five minutes or longer. The objective of this experiment were to determine whether a similar inhibition of Ca^{2+} uptake and "Ca^{2+} activated" ATPase would occur at concentrations of salyrgan, where there was less than maximal inhibition of these processes. Accordingly, the effects of varying concentrations of salyrgan on Ca^{2+} uptake and "Ca^{2+} activated" ATPase were observed. (Figure 4). In Figure 4 the Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities are expressed as per cent of control values which are measured in the absence of salyrgan. It appears that the inhibiting action of salyrgan on these two processes, is within the very narrow concentration range of 4×10^{-5} M to 6×10^{-5} M. At concentrations of salyrgan below this range there is no inhibition of either process, whereas at concentrations above this range there is 100% inhibition of both processes. As was the case with NEM, salyrgan appears to affect Ca^{2+} uptake and "Ca^{2+} activated" ATPase in a parallel fashion at the various concentration used. It is interesting to note that there is a slight stimulation

TABLE VII

Effects of NEM on Ca^{2+} uptake and ATPase activities

NEM Conc.	Ca^{2+} uptake	Ca^{2+} ATPase	basal ATPase
	µMoles Ca^{2+}/mg protein N	µMoles P_i/mg protein N	
0	3.34	2.78	.40
1×10^{-3}M	.30 (91%)	.40 (86%)	.78

Sarcoplasmic reticulum was preincubated with NEM for 35 minutes.
Control and pretreated sample were then incubated for 1 minute at 37°C.
Assay media: 2.5 mM ATP, 2.5 mM $MgCl_2$ and other substituents described in TABLE IV.
Values shown are the average obtained from duplicate assays, with those in parentheses representing the % inhibition.

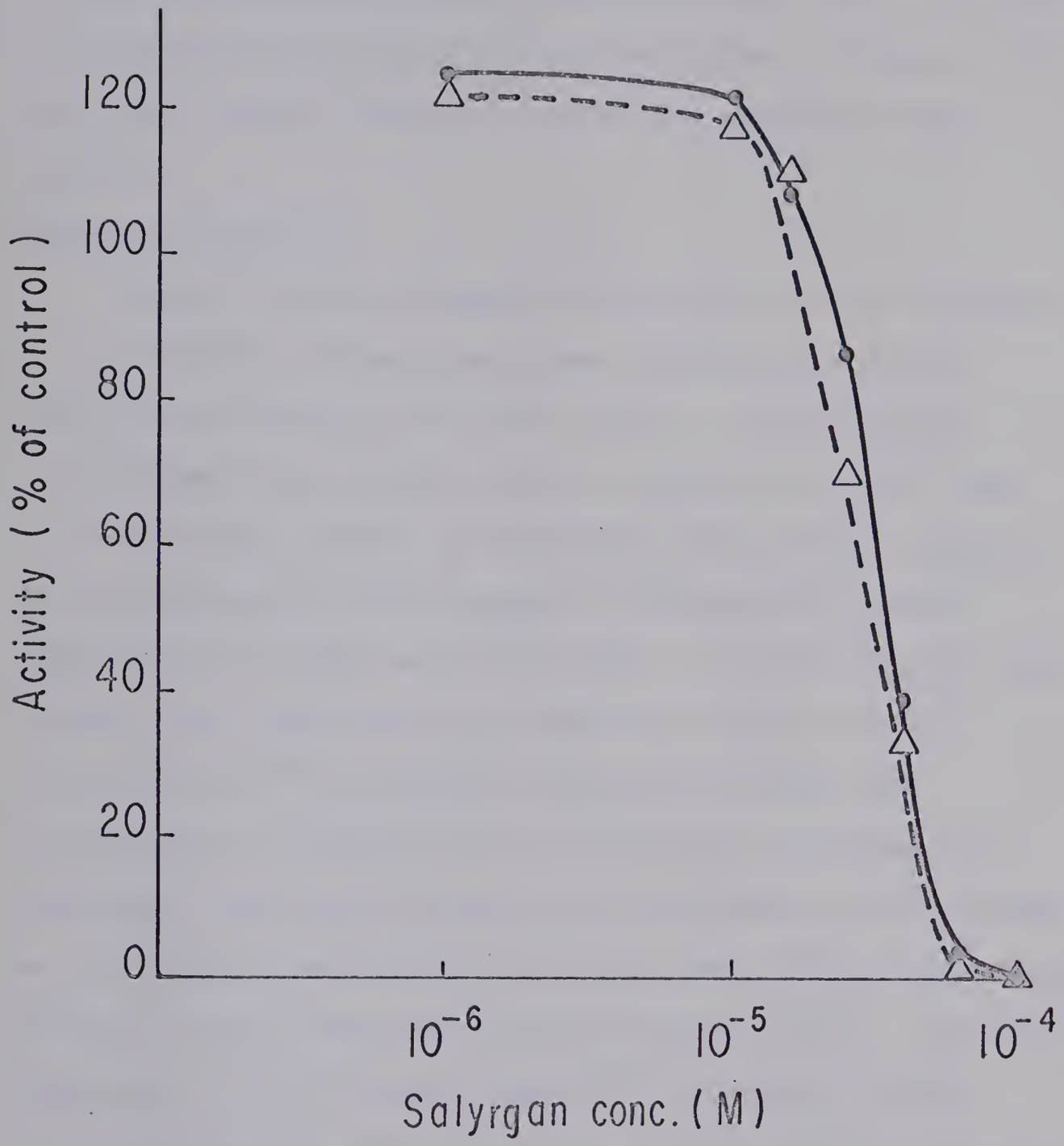

Figure 4: Effects of various concentrations of salyrgan on Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities. Sarcoplasmic reticulum samples were preincubated with salyrgan at concentrations shown for 35 minutes. Control and pretreated samples were incubated for 1 minute for Ca^{2+} uptake determinations, and for 5 minutes for "Ca^{2+} activated" ATPase activity determinations. (○-○) Ca^{2+} uptake (% of control) (△-△) "Ca^{2+} activated" ATPase activity (% of control). Assays were in duplicate.

of these processes when low concentrations (10^{-5} - 10^{-6}M) of salygran were used in the preincubation. Salyrgan did not alter "basal" ATPase at any of the concentrations tested.

Effects of Ouabain

There is much evidence which indicates that ouabain is an inhibitor of many membrane transport processes. (Bing 1965, Schultz and Curran 1970). In particular ouabain has been recently shown to inhibit the (Na^+ and K^+) - activated ATPase of an enzyme system which appears to be involved in the transport of monovalent cations (Skou 1965, Charnock and Opit 1968). In order to determine whether this agent had any comparable effects on Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities, the interaction of ouabain with sarcoplasmic reticulum was examined. There was no apparent inhibition of Ca^{2+} uptake or "Ca^{2+} activated" ATPase activity when 10^{-4} M ouabain was present in the incubation solution (Table VIII). A slight inhibition of Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities was observed when ouabain at a concentration of 2.5×10^{-4} M was preincubated with sarcoplasmic reticulum for thirty-five minutes. Basal ATPase activity was not effected by ouabain at the concentrations which were used in this experiment.

Effects of Quinidine and Procaine

Several pharmacological agents which are capable of

TABLE VIII

Effects of Ouabain on Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities

Ouabain Conc.	Ca^{2+} uptake µMoles Ca^{2+}/mg protein N	"Ca^{2+} activated" ATPase µMoles P_i/mg protein N
0	2.48	3.08
(a) 1×10^{-4}M	2.46	3.08
(b) 2.5×10^{-4}M	2.24 (10%)	2.86 (7%)

(a) Ouabain was present in the incubation solution.

(b) Sarcoplasmic reticulum was preincubated with ouabain for 35 minutes.

For assay, sarcoplasmic reticulum was incubated for 1 minute at 37°C in the following assay medium: 2.5 mM ATP, 2.5 mM $MgCl_2$ and other substituents described in TABLE IV. Values shown are the average obtained from duplicate assays, with those in parentheses representing the % inhibition.

modifying the mechanical responses of skeletal muscle, have also been found to affect the amount of calcium taken up by isolated sarcoplasmic reticulum. An example of such an agent is quinidine, which at low concentrations potentiates twitch tension, and at higher concentrations causes contracture of skeletal muscle (Lammers and Ritchie 1955) and Fuchs et al (1968) demonstrated that quinidine depressed Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities of isolated sarcoplasmic reticulum when added at a concentration which caused contraction of skeletal muscle.

Since quinidine is able to inhibit Ca^{2+} uptake and Ca^{2+} ATPase activities, it was used to examine further the relationship of these two processes. Quinidine sulfate (pH 7.0) at various concentrations was preincubated with sarcoplasmic reticulum for thirty-five minutes, and the Ca^{2+} uptake of control and pretreated samples of sarcoplasmic reticulum was measured. A 91% inhibition of Ca^{2+} uptake was observed when 10^{-3} M quinidine was preincubated with sarcoplasmic reticulum. This concentration of quinidine was used to study the inhibitory effects of this agent on Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities over three minutes.

For the measurement of ATPase activity it was necessary to remove the quinidine from the protein-free filtrate before assay, because this compound interferes with the colorimetric determination of phosphate (Ells and Faulkner

TABLE IX

Effects of quinidine on Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities

Quinidine Conc.	Ca^{2+} uptake µMoles Ca^{2+}/mg protein N	"Ca^{2+} activated" ATPase µMoles P_i/mg protein N
0	2.10	2.44
1×10^{-3}M	.10 (95%)	1.62 (34%)

Sarcoplasmic reticulum is preincubated with Quinidine for 35 minutes.
Control and pretreated samples were then incubated at 37°C for 1 minute.
Assay media: 2.5 mM, 2.5 mM $MgCl_2$ and other substituents described in TABLE IV.
Values shown are the average obtained from duplicate assays, with those in parentheses representing the % inhibition.

1963). The quinidine was adsorbed onto acid-washed Norit A charcoal. Approximately 200 mg. of charcoal were added to the filtrates of both control and quinidine treated samples.

Ca^{2+} uptake was inhibited by 95% whereas only a 34% inhibition of "Ca^{2+} activated" ATPase was observed in the presence of quinidine (Table IX). These initial results may be misleading because they did not take into account that quinidine was dissolved in a methanol solution which which may affect the function of sarcoplasmic reticulum. In a separate experiment methanol at a concentration of .625% which is the same concentration as that used in the quinidine experiment, was preincubated with sarcoplasmic reticulum. The calcium uptake observed after a one minute incubation time was not altered when methanol was present at this concentration. The "Ca^{2+} activated" ATPase activity however, was increased by 22%. Basal ATPase activities were not affected by quinidine or methanol.

Procaine

The local anesthetic procaine at a concentration of 20 mM, is able to produce contractures in some types of skeletal muscle (Issacson et al 1968). Bondani and Karler (1970) demonstrated that procaine was able to reduce the amount of calcium taken up by sarcoplasmic reticulum. They also showed that the inhibition produced by a particular concentration of procaine was increased, when

the free Ca^{2+} concentration of the incubation solution was lowered.

In our experiments a thirty-five minute preincubation of 5×10^{-2} M procaine -HCl at pH 7.0 with sarcoplasmic reticulum resulted in a 70% inhibition of Ca^{2+} uptake. When the total concentration of calcium in the incubation solution was reduced from .35 mM to .035 mM, 2.5×10^{-2} M procaine -HCl could produce the same degree of inhibition. Figures 5 and 6 represent the results of Ca^{2+} uptake and "Ca^{2+} activated" ATPase activities over three minutes in the presence and absence of procaine -HCl in the assay system. The procaine -HCl concentration used here was again 5×10^{-2} M, and the total calcium concentration of the incubation solution was .35 mM.

The Ca^{2+} uptakes observed at the various incubation times were consistently reduced by approximately 80% in the presence of procaine -HCl. The "Ca^{2+} activated" ATPase activities were also reduced in the presence of procaine -HCl, but the degree of inhibition observed varied with time and decreased from 100% after a thirty second incubation to 20% after a three minute incubation. Basal ATPase activity was not effected by procaine -HCl.

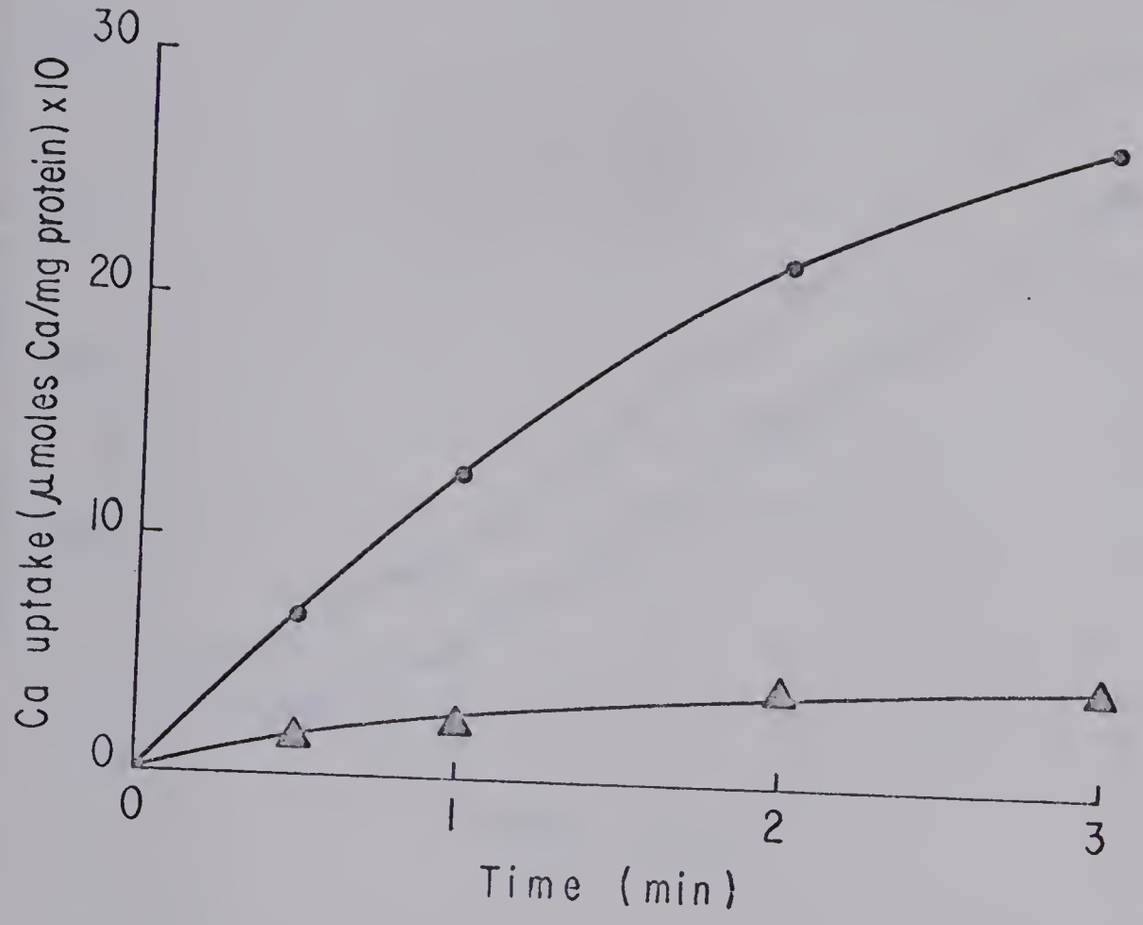

Figure 5: Effect of procaine-HCl on Ca^{2+} uptake. Sarcoplasmic reticulum was preincubated with 5×10^{-2} M procaine-HCl for 35 minutes. Control and pretreated samples were then incubated at 37°C in an assay medium containing 2.5mM ATP, and 2.5mM $MgCl_2$ and other substituents described in TABLE IV. (○ - ○) Ca^{2+} uptake of a control sample, (△ - △) Ca^{2+} uptake of a sample preincubated with procaine-HCl. Assays were in duplicate.

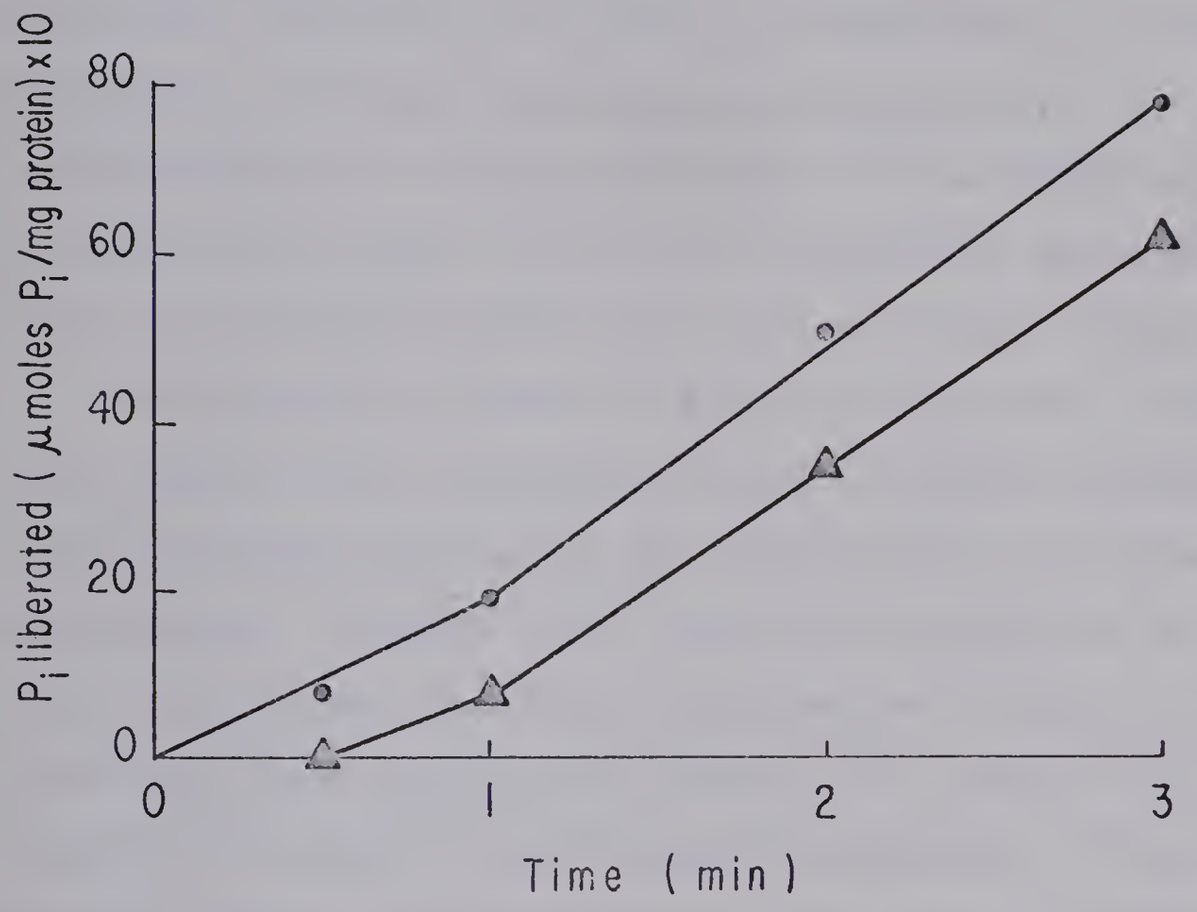

Figure 6: Effect of procaine-HCl on "Ca^{2+} activated" ATPase activity. Sarcoplasmic reticulum was preincubated with 5×10^{-2}M procaine-HCl for 35 minutes. Control and pretreated samples were then incubated at 37°C in an assay medium containing 2.5mM ATP and 2.5mM $MgCl_2$ and other substituents described in TABLE IV. (○ - ○) "Ca^{2+} activated" ATPase activity of a control sample, (△ - △) "Ca^{2+} activated" ATPase activity of a sample preincubated with procaine-HCl. Assays were in duplicate.

Effects of Temperature

The final parameter which was used to examine the degree of parallelism between Ca^{2+} uptake and "Ca^{2+} activated" ATPase was the effect of temperature on these processes. Previous experiments had revealed the concentrations of substrate ATP and Ca^{2+} ion which were necessary to provide, as closely as possible, the maximal rates for both Ca^{2+} uptake and "Ca^{2+} activated" ATPase.

For example the data of Table II show that .35 mM $CaCl_2$ added to the incubation solution provided maximal "Ca^{2+} activated" ATPase" of our sarcoplasmic reticulum preparations. Similarly the experiments described in Table X had shown that this concentration of $CaCl_2$ closely approached that required for maximal Ca^{2+} uptake when 3 mM oxalate was present in the incubation solution. Thus .35 mM $CaCl_2$ added to the incubation system would provide nearly maximal conditions for both Ca^{2+} uptake and "Ca^{2+} activated" ATPase.

It was known from the data of Table V, that when 2.5 mM ATP was added to the incubation solution, maximal "Ca^{2+} activated" ATPase activity could not be maintained for incubation periods longer than one minute. However, it was anticipated that for these temperature studies, considerably longer incubation periods than one minute would have to be employed, to ensure the generation of sufficient product, i.e. liberation of inorganic P_i from ATP, for

TABLE X

Effects of $CaCl_2$ on Ca^{2+} uptake

$CaCl_2$ Conc. µMoles/l	Ca^{2+} uptake µMole Ca^{2+}/mg protein N
.35	5.46
.45	5.20
.55	5.26
1.00	5.92

Sarcoplasmic reticulum samples were incubated for 5 minutes at 37°C.

Assay Media: variable concentrations of $CaCl_2$, 5 mM ATP, 5 mM $MgCl_2$ and other substituents described in TABLE IV. Values shown are the average of duplicate assays.

accurate colorimetric determination. Thus, further experiments were carried out which showed that at 5 mM ATP, maximal "Ca^{2+} activated" ATPase activity could be maintained for at least five minutes of incubation at 37°C (Table XI).

The times of incubation were then adjusted to allow for decreased product formation with decreasing temperature. For example from 35° down to 15°C, a five minute incubation period was employed. Figure 3 shows that under these conditions at 37°C the velocity of "Ca^{2+} activated" ATPase is approximately linear, with a slight increase in velocity after three minutes. Below temperatures of 15°C, the incubation times had to be increased to fifteen minutes to ensure adequate product formation for accurate analysis. The velocity of "Ca^{2+} activated" ATPase was maintained throughout the period of incubation.

Determinations of Ca^{2+} uptake were made from a one minute incubation time over the temperature range from 0° - 35°C. Figure 2 shows that under these conditions at 37°C, the velocity of Ca^{2+} uptake is linear. Also sufficient $^{45}Ca^{2+}$ was taken up within one minute, at any temperature from 0° - 35°C, to obviate longer incubation periods for this aspect of the study.

Since Ca^{2+} uptake and "Ca^{2+} activated" ATPase were determined after different incubation times, it was necessary to use separate aliquots of sarcoplasmic reticulum to study the effects of temperature on these processes.

TABLE XI

Effects of ATP on "Ca^{2+} activated" ATPase activity

ATP Conc. mM/l	Ca^{2+} ATPase μ Moles P_i/mg protein N
2.5	21.22
5.0	30.52
10.0	30.78

Sarcoplasmic reticulum samples were incubated for 5 minutes at 37°C.

Assay media: variable concentration of ATP and $MgCl_2$ [ATP = $MgCl_2$], and other substituents described in TABLE IV.

Values shown are the average of triplicate assays.

However, in any one experiment the whole temperature range was tested on a single sarcoplasmic reticulum preparation.

The effects of temperature on the rates of Ca^{2+} uptake, "Ca^{2+} activated" ATPase, and "basal" ATPase are illustrated in Table XII. The rates of these processes are increased when the temperature is elevated, however; "basal" ATPase is less sensitive to the effects of temperature than either Ca^{2+} uptake or "Ca^{2+} activated" ATPase. Arrhenius plots were constructed using the data of Table XII, where the log of the rate of product formation, that is either the liberation of inorganic phosphate or Ca^{2+} ion taken up, was plotted as a function of the reciprocal of the absolute temperature. The Arrhenius plots shown in Figures 7, 8 and 9 are typical of those obtained for all other sarcoplasmic reticulum preparations examined.

The Arrhenius plot for Ca^{2+} uptake (Figure 7) is linear over the temperature range of 0° - 35°C. In contrast the Arrhenius plot for "Ca^{2+} activated" ATPase (Figure 8), is clearly not linear over this same temperature range. The plot shows a point of inflection at about 10°C, and is approximately linear at temperatures above and below 10°C. An examination of the slopes of the Arrhenius plot below and above 10°C, shows that the slope is greater below 10°C. Therefore the apparent activation energy of the "Ca^{2+} activated" ATPase process is also greater at temperatures

TABLE XII

Effects of Temperature on Ca^{2+} uptake and ATPase activities

Temp °C	Ca^{2+} uptake µMole Ca^{2+}/mg protein N/hr	Ca^{2+} ATPase µMoles P_i/mg protein N/hr	"basal" ATPase µMoles P_i/mg protein N/hr
35	216.60	209.72	65.22
30	150.26	135.00	56.28
25	100.86	92.82	46.44
20	62.42	58.68	34.26
15	37.18	32.10	27.92
10	21.66	16.54	16.16
8	16.18	11.78	13.96
6	13.80	8.82	11.38
4	11.16	5.52	9.54
2	8.38	3.70	7.32
0	6.26	1.84	4.76

Typical values obtained with sarcoplasmic reticulum preparations after storage at -15°C for a maximum of two weeks. All Ca^{2+} uptake values obtained from a single preparation. Values for Ca^{2+} ATPase and "basal" ATPase were obtained from a separate preparation. This data was used to calculate the Arrhenius plots given in Figures 7, 8 and 9. Assay conditions are described fully in text. Values shown are means of 4 determinations.

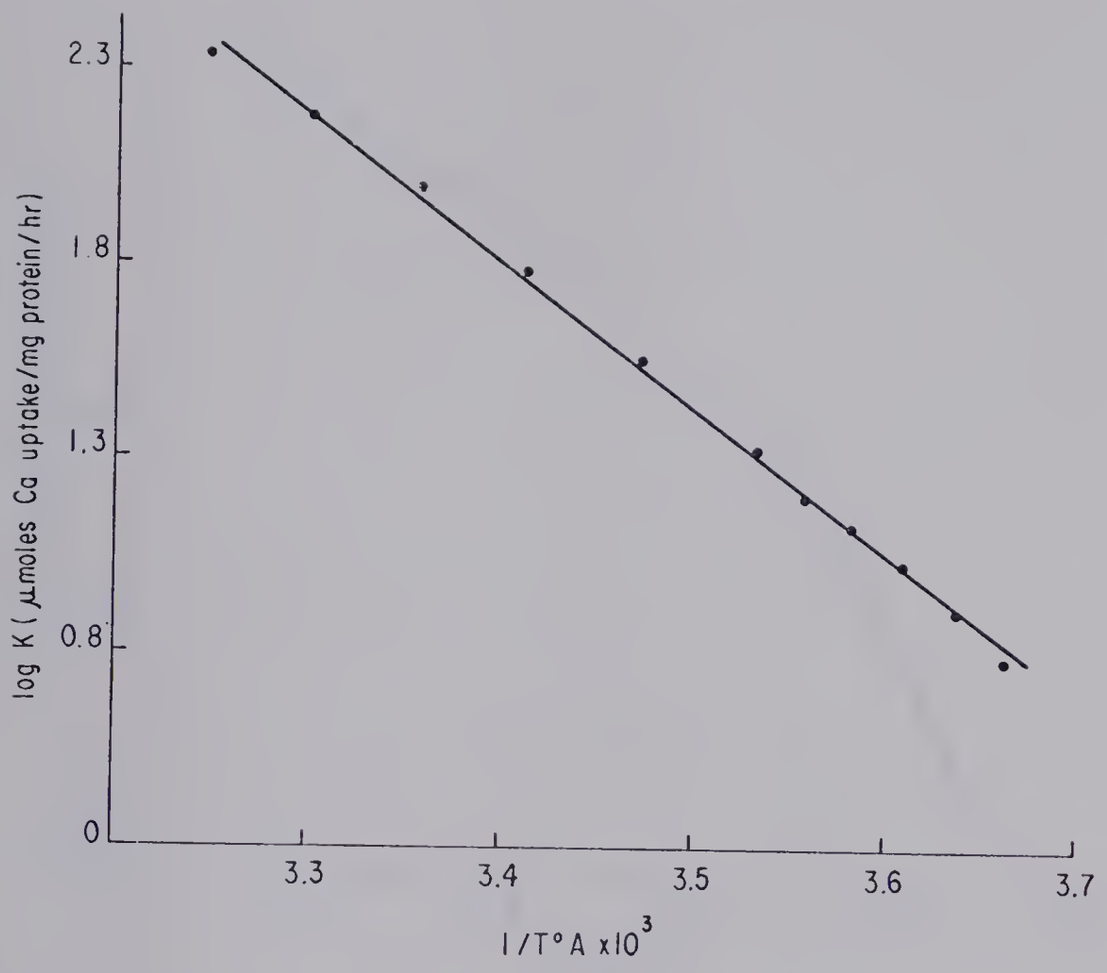

Figure 7: An Arrhenius plot of Ca^{2+} uptake. Assay conditions are described fully in text. Data for plot is obtained from TABLE XII. The apparent energy of activation is 17.0 K calories/mole over the range of 0° - 35°C.

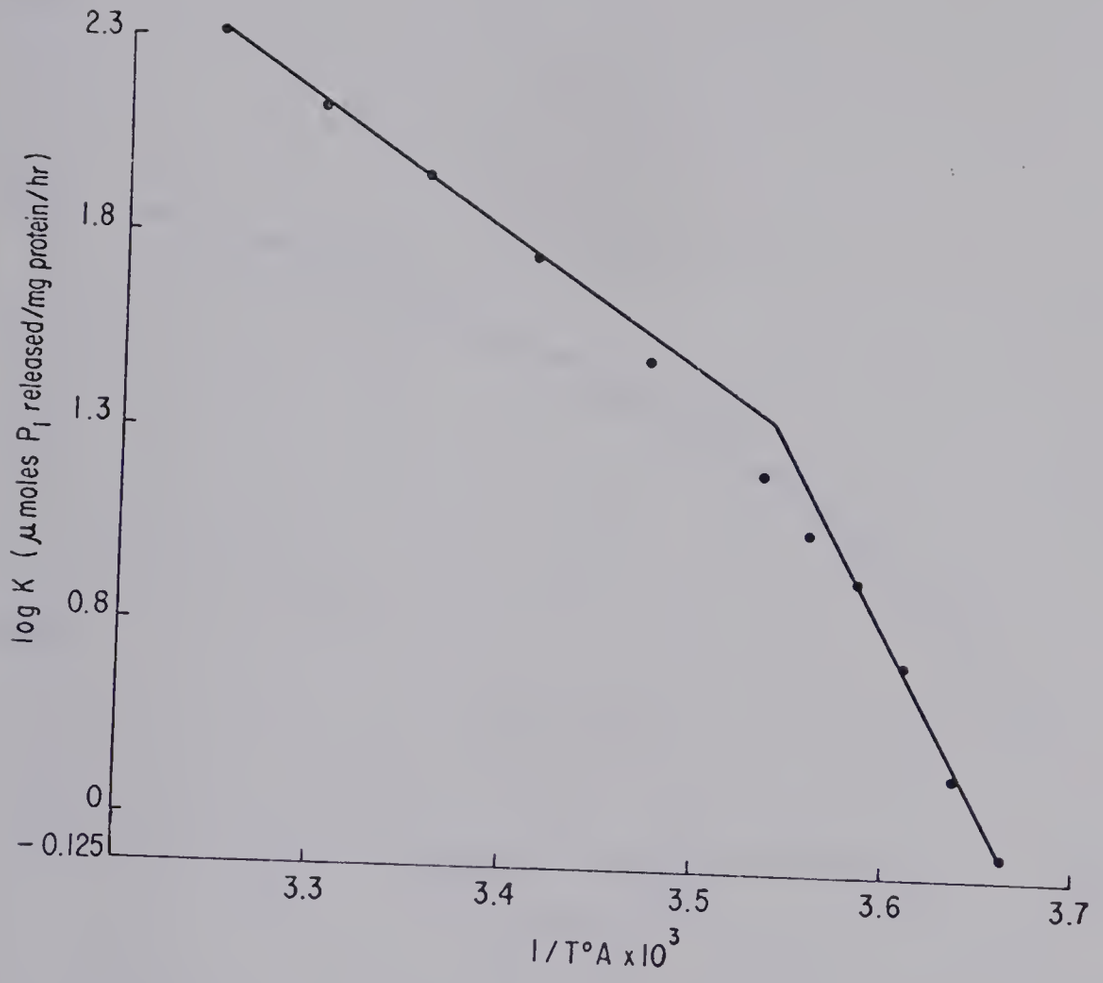

Figure 8: An Arrhenius plot of "Ca^{2+} activated" ATPase. Assay conditions are described fully in text. Data for plot is obtained from TABLE XII. The apparent energy of activation is 33.4 K calories/mole over the range of 0° - 10°C, and 16.3 K calories/mole over the range of 15° - 35°C.

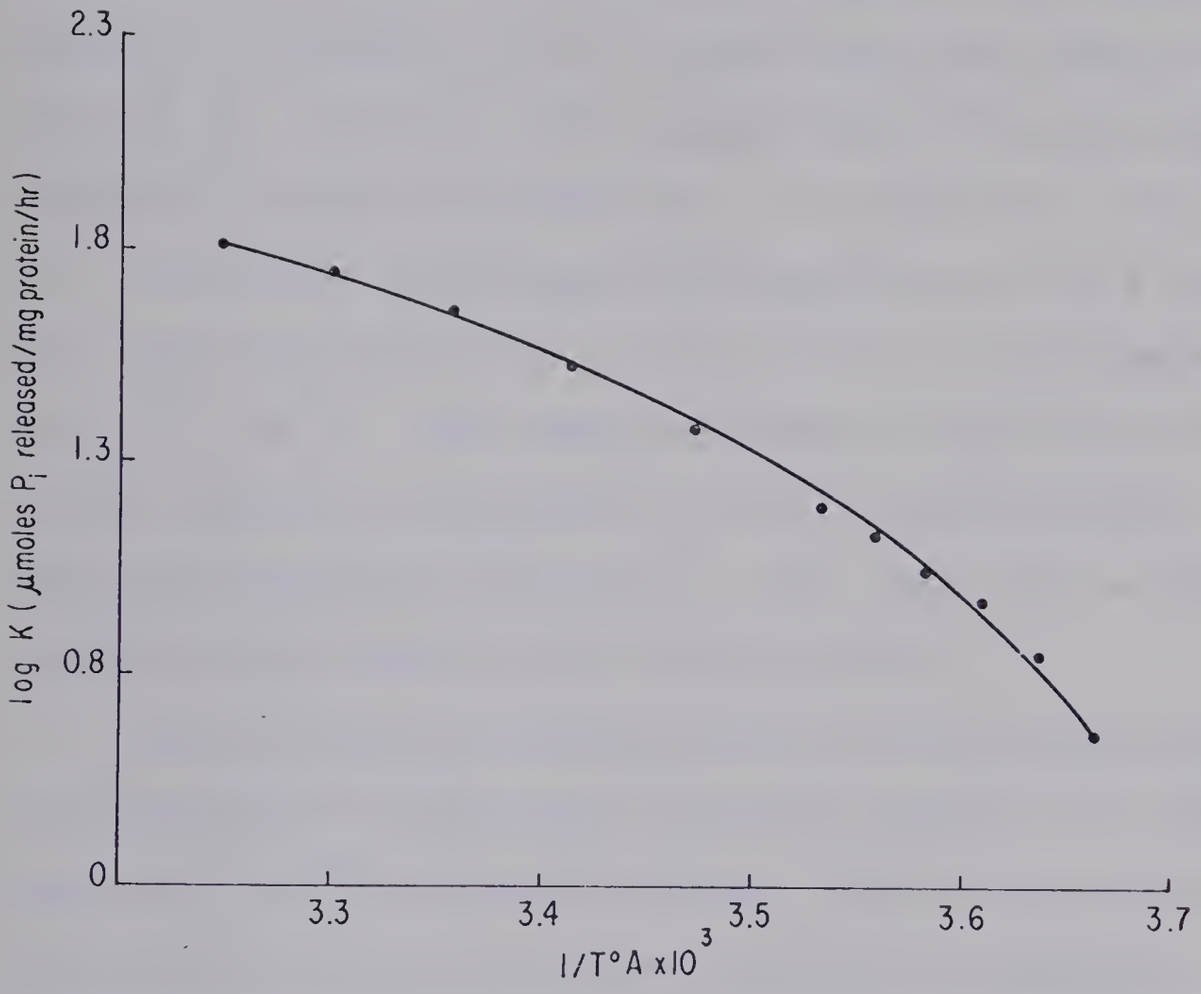

Figure 9: An Arrhenius plot of "basal" ATPase. Assay conditions are described fully in text. Data for plot is obtained from TABLE XII. The apparent energy of activation is 12.5 K calories/mole over the range of 0° - 35°C.

below 10°C. The Arrhenius plot for "basal" ATPase (Figure 9) appears to be neither linear or discontinuous but is curvilinear in form. The estimated value of the apparent energy of activation for Ca^{2+} uptake over the temperature range of 0° - 35°C for three preparations of sarcoplasmic reticulum is 18.2 K calories Mole. In contrast, values for "Ca^{2+} activated" ATPase were estimated to be 32.3 K calories/mole over the range of 0° - 10°C and 16.1 K calories/mole over 15° - 35° C. The apparent energy of activation of "basal" ATPase was estimated to be 12.3 calories/mole over the temperature range of 0° - 35°C, but must be doubtful because of the shape of the Arrhenius plot.

The different characteristics of the Arrhenius plots, and differences in apparent activation energies for Ca^{2+} uptake and "Ca^{2+} activated" ATPase, indicate that these two processes do not have the same temperature dependence. This finding suggests that there is a possible dissociation of Ca^{2+} uptake from "Ca^{2+} activated" ATPase.

Calculations of the values of the apparent energies of activation were facilated by the use of the APL/360 computing system of the University of Alberta. The process involved the fitting of a linear regression line by the method of least squares to points whose co-ordinates are the reciprocal of the absolute temperature, and the log of the rate of Ca^{2+} uptake or "Ca^{2+} activated" ATPase. The slope of the linear regression line is multiplied by 4.576, to provide the apparent energy of activation.

TABLE XIII

Apparent energy of activation of Ca^{2+} uptake

SR Preparation	No. of determinations	*Apparent Energy of activation 0° - $35^{\circ}C$
3	3	17.0 ± .3
4	4	17.6 ± .3
5	4	20.1 ± 1.0

Sarcoplasmic reticulum samples were incubated for 1 minute at all temperatures studied.

Assay media: as described in TABLE IV.

Values for apparent energies of activation are mean K calories/mole ± S.E.

TABLE XIV

Apparent energy of activation of "Ca^{2+} activated" ATPase and "basal" ATPase

SR Preparation	No. of determinations	Apparent Energy of activation		
		"Ca^{2+} activated" ATPase		"basal" ATPase
		0° - 10°C	15° - 35°C	0° - 35°C
1	4	31.2 ± 1.8	15.9 ± 1.0	12.1 ± .8
2	4	33.4 ± 2.4	16.3 ± .7	12.5 ± 1.2

Sarcoplasmic reticulum samples were incubated for 5 minutes at 15° - 35°C, and for 15 minutes at 0° - 10°C.

Assay media: as described in TABLE IV

Values for apparent energies of activation are mean K calories/mole ± S.E.

Discussion

The objectives of these experiments were to examine the degree of parallelism between Ca^{2+} uptake and "Ca^{2+} activated" ATPase of sarcoplasmic reticulum. An approach which was used involved the study of the effects of various types of chemical agents on Ca^{2+} uptake and "Ca^{2+} activated" ATPase activity. The rationale for using this approach, was that if these agents effected Ca^{2+} uptake and "Ca^{2+} activated" ATPase in a similar manner, this may indicate that these two processes are closely related.

When sarcoplasmic reticulum is preincubated with the sulfhydryl reagents NEM or salygran Ca^{2+} uptake and "Ca^{2+} activated" ATPase activity are inhibited in a parallel manner. No explanation can be offered for the apparent slight stimulation of these two processes when low concentrations (10^{-6} to 10^{-5} M) salygran were used for the preincubation. The lack of effect of these agents on "basal" ATPase may indicate, as has been suggested by Hasselbach (1964b), that "basal" ATPase and "Ca^{2+} activated" ATPase are separate enzyme systems.

The results of the above experiments indicate that sulfhydryl groups of sarcoplasmic reticulum are important functional groups for both the uptake of Ca^{2+} and "Ca^{2+} activated" ATPase.. This is in accordance with the conclusions made by Hasselbach and Seraydarian (1966). It appears that these sulfhydryl reagents inhibit "Ca^{2+} activated" ATPase by decreasing the formation of a phospho-

membrane complex (Makinose 1970), which as previously discussed in the "Introduction" has been implicated as an intermediate in the "Ca^{2+} activated" ATPase process of sarcoplasmic reticulum.

Although studies with these sulfhydryl reagents suggest that Ca^{2+} uptake and "Ca^{2+} activated" ATPase are closely related processes, the nature of this relationship cannot be determined from these experiments. For example, it cannot be determined whether inhibition of "Ca^{2+} activated" ATPase by the sulfhydryl reagents, merely eliminates the energy source required for Ca^{2+} accumulation, or whether this is an actual inhibition of the Ca^{2+} transporting enzyme.

When quinidine sulfate at a concentration of 10^{-3} M was preincubated with sarcoplasmic reticulum, both Ca^{2+} uptake and "Ca^{2+} activated" ATPase activity were again inhibited (Table IX). However, Ca^{2+} uptake was inhibited to a larger extent than "Ca^{2+} activated" ATPase. The degree of inhibition of Ca^{2+} uptake was also greater than than observed for "Ca^{2+} activated" ATPase when procaine -HCl at a concentration of 5×10^{-2} M was used in the preincubation. For example when sarcoplasmic reticulum was preincubated with procaine -HCl the Ca^{2+} uptakes observed at the various incubation times were reduced by approximately 80%, whereas the degree of "Ca^{2+} activated" ATPase inhibition varied with time and decreased from 100% after a thirty second

incubation to 20% after a three minute incubation (Figures 5 and 6).

In contrast to the findings for the sulfhydryl reagents NEM and salygran, the data for experiments with quinidine sulfate and procaine -HCl suggest that there may be partial uncoupling of Ca^{2+} uptake from "Ca^{2+} activated" ATPase. However, one must exercise considerable caution in the interpretation of these and other results of a similar nature. For example it has been shown that agents such as diethyl ether (Inesi et al 1967) and oleic acid (Mortinosi 1964) when used at certain concentrations reduce the Ca^{2+} uptake observed but do not affect "Ca^{2+} activated" ATPase. Fiehn and Hasselbach (1969) observed that when sarcoplasmic vesicles loaded with calcium oxalate are suspended in a solution of diethyl ether (concentration of diethyl ether used is one which almost completely suppresses Ca^{2+} uptake observed, but does not effect "Ca^{2+} activated" ATPase), calcium is released much more rapidly than in a solution free of ether. Thus it would appear that diethyl ether makes the vesicular membranes leaky so that their ability to store calcium is abolished. Thus although the net calcium uptake is decreased by diethyl ether, Ca^{2+} transport, is not necessarily inhibited. Therefore it is important to note that the apparent dissociation between Ca^{2+} uptake and "Ca^{2+} activated" ATPase which is observed with agents such as diethyl ether which increase the vesicular membrane

permeability to Ca^{2+}, cannot be explained on the basis of there being a "true" uncoupling of these two processes.

Although no evidence is provided in our experiments, it is conceivable that quinidine and procaine act in a similar manner to diethyl ether and reduce Ca^{2+} uptake by increasing the membrane permeability to calcium. A comparison of the efflux of Ca^{2+} from Ca^{2+} oxalate loaded sarcoplasmic vesicles suspended in procaine and quinidine solutions, with those suspended in the absence of these agents could provide more information on how these reagents act to dissociate Ca^{2+} uptake and "Ca^{2+} activated" ATPase.

This inhibiting effects of these agents on "Ca^{2+} activated" ATPase may be similar to that exerted by diethyl ether which also inhibits "Ca^{2+} activated" ATPase at high concentrations (Fiehn and Hasselbach 1969).

Our studies with sulfhydryl reagents indicate that Ca^{2+} uptake and "Ca^{2+} activated" ATPase are closely related processes, but as was previously described the nature of this relationship cannot be determined from these or other similar experiments. The experiments with quinidine and procaine -HCl suggest the possibility of an uncoupling of Ca^{2+} uptake from "Ca^{2+} activated" ATPase; however, further experiments such as those suggested above are necessary to determine whether this observed dissociation is due to an action of these agents which increases the permeability of the sarcoplasmic vesicles to calcium.

Thus the experimental results obtained from studies such as the ones described here must be very carefully interpreted, and furthermore they can only provide a limited amount of information on the nature of the relationship between Ca^{2+} and "Ca^{2+} activated" ATPase.

The effects of temperature on Ca^{2+} uptake and "Ca^{2+} activated" ATPase were observed, in order to further examine the degree of parallelism between these two processes. The concentrations of substrate ATP and Ca^{2+} ion which were used and the times of incubation were adjusted as described in "Results", to provide conditions in any one experiment which were as closely as possible optimal for the Ca^{2+} uptake and "Ca^{2+} activated" ATPase processes. The rationale for the procedures was to eliminate variables other than temperature which could affect Ca^{2+} uptake or "Ca^{2+} activated" ATPase. For example, Hasselbach and Makinose (1962) demonstrated that with 5 mM ATP as an energy donor, 2 mM ADP produced 50% inhibition of Ca^{2+} uptake and "Ca^{2+} activated" ATPase.

The increases in the rates of Ca^{2+} uptake and "Ca^{2+} activated" ATPase which are observed with elevations of temperature (Table XII) illustrate that both these processes are very temperature dependent. Arrhenius plots of Ca^{2+} uptake and "Ca^{2+} activated" ATPase were constructed to further examine the temperature dependence of these two processes. The Arrhenius plot for Ca^{2+} uptake is linear over the temperature range of 0° - $35^{\circ}C$, and apparent energy of

activation of this process is about 18.2 K calories/mole. In contrast the Arrhenius plot of "Ca^{2+} activated" ATPase is discontinuous and appears to show a point of inflection at about 10°C. The plot is approximately linear from 15° - 35°C, and 0° - 10°C and the apparent energies of activation over these temperature ranges are about 16.1 K calories/mole and 32.3 K calories/mole respectively. These data indicate that Ca^{2+} uptake and "Ca^{2+} activated" ATPase do not have the same temperature dependence. The differences in the Arrhenius plots and apparent activation energies of "Ca^{2+} activated" ATPase and "basal" ATPase provides additional indirect evidence that two separate enzyme systems may be involved in the hydrolysis of ATP by sarcoplasmic reticulum.

Our results on the effects of temperature appear to be at variance with those obtained by Inesi and Watanabe (1967). Inesi and Watanabe (1967) reported that the Arrhenius plots of Ca^{2+} uptake and "Ca^{2+} activated" ATPase were linear and that the energies of activation of both processes were similar. For example these workers reported that the energy of activation for Ca^{2+} uptake was about 20.4 - 30.0 K calories/mole, and the energy of activation for "Ca^{2+} activated" ATPase was approximately 22.6 - 26.8 K calories/mole. However in their experiments Ca^{2+} uptake and "Ca^{2+} activated" ATPase were determined at only four temperatures over the narrow range of 5° - 20°C. Consequently their data can be fitted by a variety of plots. Therein

may be the reason for the observed differences when our results are compared to their's.

Our experiments on the effects of temperature illustrate that Ca^{2+} uptake and "Ca^{2+} activated" ATPase exhibit different temperature dependencies, which suggests that Ca^{2+} uptake may be capable of dissociation from "Ca^{2+} activated" ATPase in some unknown manner. Although it is tempting to speculate from these experimental results about the role of "Ca^{2+} activated" ATPase in the calcium accumulating process, at least several different interpretations can be made. One possibility is that the phospho-membrane complex which has been implicated as an intermediate step during the hydroysis of ATP by "Ca^{2+} activated" ATPase, also functions as the carrier of Ca^{2+} to enable sarcoplasmic reticulum preparations to accumulate Ca^{2+} against a concentration gradient.

However, this suggestion implies that the phospho-membrane complex is an intermediate common to both the "Ca^{2+} activated" ATPase and Ca^{2+} uptake processes. This hypothesis would appear to be inconsistent with our findings of the different temperature dependencies of these processes. Another possibility is that the "Ca^{2+} activated" ATPase energizes separate sites on the sarcoplasmic reticulum membrane, through a phosphorylated intermediate by perhaps a transfer of phosphate or by some inductive process. These "energized" sites could then function as Ca^{2+} carriers to

enable the sarcoplasmic reticulum to accumulate Ca^{2+} against a concentration gradient.

Such a hypothesis may better account for the apparent dissociation of Ca^{2+} uptake from "Ca^{2+} activated" ATPase by variable responses to changes in temperature. However, our studies are indirect and can only provide a limited understanding of the relationship between Ca^{2+} uptake and "Ca^{2+} activated" ATPase.

Very recently a seemingly more direct approach has been utilized by MacLennan et al (1970, 1971). These workers have prepared a highly purified "Ca^{2+} activated" ATPase from sarcoplasmic reticulum, which although of high specific activity, possessed a low Ca^{2+} binding capacity (MacLennan 1970). However, they have also have been able to prepare from sarcoplasmic reticulum a separate protein devoid of "Ca^{2+} activated" ATPase activity, but with a binding capacity for Ca^{2+}, such that Ca^{2+} binding to this protein could account for a large proportion of Ca^{2+} accumulated by the sarcoplasmic reticulum. (MacLennan and Wong 1971). Their experiments indicate that this protein ("Calsequestrin") is localized in the interior of the vesicular membranes. It is their interpretation that they have achieved a physical separation of the energy transduction and ion translocation steps ("Ca^{2+} activated", ATPase) from the Ca^{2+} ion binding sites (Calsequestrin).

Their findings suggest that there may be a partial

dissociation of the overall Ca^{2+} accumulation process, and that this process is the function of two separate systems which could quite conceivably possess different responses to temperature. Nevertheless, as MacLennan (1970) proposes that "Ca^{2+} activated" ATPase is the total ion translocation step, it is still difficult to see how different temperature effects could be obtained, unless his alternative suggestion for the involvement of a further and as yet unidentified component is correct.

BIBLIOGRAPHY

Aldridge, W. N., (1962). Adenosine triphosphate in the microsomal fraction from rat brain. Biochem. J. 83, 527-533.

Bendall, J. R. (1952). Effect of the "Marsh" factor on the shortening of muscle fibre models in the presence of adenosine triphosphate. Nature 170, 1058-1060.

Bendall, J. R., (1953). Further observations on a factor (the "Marsh" factor) effecting relaxation of ATP-shortened muscle fibre models and the of Ca and Mg ions upon it. J. Physiol. 121, 232-254.

Bing, R.J., (1965). Cardiac metabolism. Physiol. Rev. 45, 171-213.

Bondani, A. and Karler, R. (1970). Interaction of calcium and local anesthetics with skeletal muscle microsomes. J. Cell. Physiol. 75, 192-211.

Bozler, E. (1954). Relaxation in extracted muscle fibres. J. Gen. Physiol. 38 , 149-159.

Briggs, F. N., and Fuchs, F., (1960). Biosynthesis of a muscle relaxing substance. Biochim. Biophys. Acta. 42, 519-527.

Carvahlo, A. P., (1966). Binding of cations by microsomes from rabbit skeletal muscle. J. Cell. Physiol. 67, 73-84.

Carvahlo, A. P., and Leo, B., (1967). Effects of ATP on the interaction of Ca^{++}, Mg^{++}, and K^{+} with fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle. J. Gen. Physiol. 50, 1327-1352.

Charnock, J. S., and Opit, L. J. (1968). Membrane metabolism and ion transport. In The Biological Basis of Medicine, edited by E.E. Bittar, Vol. 1, Academic Press, London and New York, p. 69-103.

Constantin, L. L., Franzini-Armstrong, C., and Podolsky, R. J. (1965) Localization of calcium accumulating structures in striated muscle fibres. Science 147, 158-160.

Ebashi, F. (1961). Does EDTA bind to actomyosin? J. Biochem. 50, 77-78.

Ebashi, S. (1960). Calcium binding and relaxation in the actomyosin system. J. Biochem. 50, 236-244.

Ebashi, S. (1961). Calcium binding activity of vesicular relaxing factor. J. Biochem. 50, 236-244.

Ebashi, S. (1965). The sarcoplasmic reticulum and excitation - contraction coupling. In Molecular Biology of Muscular Contraction, edited by S. Ebashi, F. Oosawa, T. Sekine and Y. Tonomura. B.B.A. Library, Vol. 9, Igaku-Shoin, Tokyo, and Elsevier Publishing Co., Amsterdam, p. 197-206.

Ebashi, S., and Endo, M. (1968). Calcium ion and muscle contraction. Prog. Biophys. Mol. Biol. 18, 123-183.

Ebashi, S., and Lipmann, F. (1962). Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. J. Cell. Biol. 14, 389-400.

Ells, H. A., and Faulkner, P. (1963). The effect of quinidine on some enzymes which hydrolyze adnosine triphosphate. Arch. Int. Pharmacodyn. 141, 75-82.

Fiehn, W., and Hasselbach, W. (1969). The effect of diethyl ether upon the function of vesicles of sarcoplasmic reticulum. Europ. J. Biochem. 9, 574-578.

Fiske, C. H., and Subba Row, Y. (1925). The colorimetric determination of phosphorus. J. Biol. Chem. 66, 375-400.

Fuchs, F., Gertz, E. W., and Briggs, F. N. (1968). The effects of quinidine on calcium accumulation of isolated sarcoplasmic reticulum of skeletal and cardiac muscle. J. Gen. Physiol. 52, 955-968.

Hasselbach, W. (1964a). Relaxation and the sarcotubular calcium pump. Fed. Proc. 23, 909-912.

Hasselbach, W. (1964b). Relaxing factor and the relaxation of muscle. Prog. Biophys. Mol. Biol. 14, 167-222.

Hasselbach, W. (1966). Structural and enzymatic properties of calcium transporting membranes of the sarcoplasmic reticulum. Annal N.Y. Acad. Sci. 137, 1041-1048.

Hasselbach, W., and Makinose, M. (1961). Die calciumpumpe der "Erschalaffungsgrana" des muskels unde ihre abhangigkeit der ATP-spaltung. Biochem. Z. 333, 518-528.

Hasselbach, W., and Makinose, M. (1962). ATP and active transport. Biochem. Biophys. Res. Comm. 7, 132-136.

Hasselbach, W., and Makinose, M. (1963). Ueber den mechanismus des calciumtransportes durch die membranen des sarkoplasmatichen reticulums. Biochem. 2. 339, 94-111.

Hasselbach, W., and Seraydorian, K. (1966). The role of sulfhydryl group in calcium transport through the sarcoplasmic membranes of skeletal muscle. Biochem. 2. 345, 159-172.

Hasselbach, W., and Weber, H. H. (1953). Der einfluss des M B - faktors auf die kontrakion des fasemodels. Biochim. Biophys. Acta 11, 160-161.

Heilbrunn, L. V., and Wiercinski, F. J. (1947). The action of various cations on muscle protoplasm. J. Cell. Comp. Physiol. 29, 15-32

Hill, A. V. (1949). The abrupt transition from rest to activity in muscle. Proc. Roy. Soc. (London) Ser. B, 136, 399-420.

Hodgkin, A. L., and Horowicz, P. (1960). Potassium contractures in single muscle fibres. J. Physiol. 153, 386-403.

Howell, J. N. (1969). A lesion of transverse tubules of skeletal muscle. J. Physiol. 201, 515-533.

Huxley, H. E. (1964). Evidence for continuity between the central elements of the triads and the extracellular space in frog sartorius muscle. Nature 202, 1067-1071.

Huxley, A. F., and Taylor, R. E. (1958). Local activation of striated muscle fibres. J. Physiol. 144, 426-441.

Inesi, G., Goodman, J. J., and Watanabe, S. (1967). Effect of diethyl ether on the adenosine triphosphatase activity and calcium uptake of fragmented sarcoplasmic reticulum of rabbit skeletal muscle. J. Biol. Chem. 242, 4637-4643.

Inesi, G., Maring, E., Murphy, A. J., and McFarland, B. H. (1970). A study of the phosphorylated intermediate of sarcoplasmic reticulum. Arch. Biochem. Biophys. 138, 285-294.

Inesi, G., and Watanabe, S. (1967). Temperature dependence of ATP hydrolysis and calcium uptake by fragmented sarcoplasmic membranes. Arch. Biochem. Biophys. 121, 665-671.

Isaacson, A., Taylor, S. R., and Hinkes, M. J. (1968). Contracture and potentiation of fast and slow muscles of the rat - effects of quinine, procaine, caffeine and zinc. Fed. Proc. 28, 443.

Lammers, W., and Ritchie, J. M. (1955). The actions of quinine and quinidine on the contractures of striated muscle. J. Physiol. (London) 129, 412-423.

Kielley, W. W., and Myerhoff, O. (1948). A new magnesium-activated adenosine triphosphatase from muscle. J. Biol. Chem. 174, 387-388.

Kielley, W. W., and Myerhoff, O. (1949). Studies of the adenosinetriphosphatase of muscle II. J. Biol. Chem. 176, 591-601.

Landgraf, W. C., and Inesi, G. (1969). ATP dependent conformational change in "skin labelled" sarcoplasmic reticulum. Arch. Biochem. Biophys. 130, 111-118.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

MacLennan, D. H. (1970). Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. J. Biol. Chem. 245, 4508-4518.

MacLennan, D. H., Seeman, P., Iles, G. H., and Yip, C.C. (1971). Membrane formation by the adenosine triphosphatase of sarcoplasmic reticulum. J. Biol. Chem. 246, 2702-2710.

MacLennan, D. H., and Wong, P. T. S. (1971). Isolation of a calcium sequestering protein from sarcoplasmic reticulum. Proc. Nat. Acad. Sci. U.S.A. 68, 1231-1235.

Makinose, M. (1966). Die nucleosidtriphosphat-nucleosiddephosphat-transphosphorylose-activitat der vesikels des sarcoplasmotischen reticulums. Biochem. Z. 345, 80-86.

Makinose, M. (1969). The phosphorylation of the membranal protein of the sarcoplasmic vesicles during active calcium transport. Europ. J. Biochem. 10, 74-82.

Marsh, B. B. (1951). A factor modifying muscle fibre syneresis. Nature 167, 1065-1066.

Marsh, B. B. (1952). The effects of adenosine triphosphate on the fibre volume of a muscle homogenate. Biochem. Biophys. Acta. 9, 247-260.

Martinosi, A. (1964). Role of phospholipids in ATPase activity and Ca transport of fragmented sarcoplasmic reticulum. Fed. Proc. 23, 913-921.

Martinosi, A. (1968). Sarcoplasmic reticulum IV. Solubilization of microsomal adenosine triphosphatase. J. Biol. Chem. 243, 71-81.

Martinosi, A. (1969). Sarcoplasmic reticulum VII. Properties of a phosphoprotein intermediate implicated in calcium transport. J. Biol. Chem. 244, 613-620.

Martinosi, A., and Feretos, R. (1964a). Sarcoplasmic reticulum I. The uptake of Ca^{++} by sarcoplasmic reticulum fragments. J. Biol. Chem. 239, 648-658.

Martinosi, A., and Feretos, R. (1964b). Sarcoplasmic reticulum II. Correlation between adenosine triphosphatase activity and Ca^{++} uptake. J. Biol. Chem. 239, 659-668.

Muscatello, U., Andersson-Cedergren, E., and Azzone, G. F. (1962). The mechanism of muscle fibre relaxation adenosine triphosphatase and relaxing activity of the sarcotubular system. Biochem. Biophys. Acta. 63, 55-74.

Onishi, T., and Ebashi, S. (1964). The velocity of calcium binding of isolated sarcoplasmic reticulum. J. Biochem. 55, 599-603.

Parker, C. J., and Gergely, J. (1960). Soluble relaxing factor from muscle. J. Biol. Chem. 235, 3449-3453.

Peachey, L. D. (1965). The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. J. Cell. Biol. 25, Part II, 209-231.

Peachey, L. D., and Huxley, A. F. (1962). Structural identification of twitch and slow striated muscle fibres of the frog. J. Cell. Biol. 13, 177-180.

Peachey, L. D., and Porter, K. R. (1959). Intracellular impulse conduction in muscle cells. Sci. 129, 721-722.

Porter, K. R., and Palade, G. E. (1957). Studies of the endoplasmic reticulum III. Its form and distribution in striated muscle cells. J. Biophys. Biochem. Cytol. 3, 269-300.

Portzehl, H. (1957). Die bindung des Erschloffungsfaktors on die muskelgrana. Biochim. Biophys. Acta. 42, 519-527.

Portzehl, H., Caldwell, P. C., and Ruegg, J. C. (1964). The dependence of contraction and relaxation of muscle fibres from the crab Maia squinado on the internal concentration of free calcium ions. Biochim. Biophys. Acta. 79, 581-591.

Schultz, S. G., and Curran, P. F. (1970). Coupled transport of sodium and organic solutes. Physiol. Rev. 45, 637-717.

Seidel, J. C., and Gergely, J. (1964). Studies on myofibrillar adenosine triphosphatase with calcium-free adenosine triphosphate. J. Biol. Chem. 239, 3331-3335.

Skou, J. C. (1957). Influence of some cations on an adensoine triphosphatase from peripheral nerves. Biochim. Biophys. Acta 23, 394-401.

Skou, J. C. (1965). Enzymatic basis for active transport of Na and K across cell membrane. Physiol. Rev. 45, 596-617.

Smith, D. S. (1966). The organization and function of the sarcoplasmic reticulum and T-system of muscle cells. Progr. Biophys. Mol. Biol. 16, 107-142.

Watanabe, S. (1955). Relaxing effects of EDTA on glycerol-treated muscle fibres. Arch. Biochem. Biophys. 54, 559-562.

Weber, A. (1966a). Energized calcium transport and relaxing factors. In Current Topics in Bioenergetics edited by D. R. Sonadi, Academic Press, New York and London.

Weber, A., Herz, R., and Reiss, I. (1966b). Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. Biochem . 345, 329-369.

Weber, A., and Herz, R. (1963). The binding of calcium to actomyosin systems in relation to their biological activity. J. Biol. Chem. 238, 599-605.

Weber, A., Herz, R., and Reiss, I. (1963). On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum. J. Gen. Physiol. 46, 679-702.

Winegrad, S. (1965). Autoradiographic studies of intracellular calcium in frog skeletal muscle skeletal muscle. J. Gen. Physiol. 48, 455-479.

Winegrad, S. (1968). Intracellular calcium movements of frog skeletal muscle during recovery from tetanus. J. Gen. Physiol. 51, 65-83.

Yamamoto, T., and Tonomura, Y. (1967). Reaction mechanism of the Ca^{2+}- dependent ATPase of sarcoplasmic reticulum from skeletal muscle. J. Biochem. 62, 558-575.